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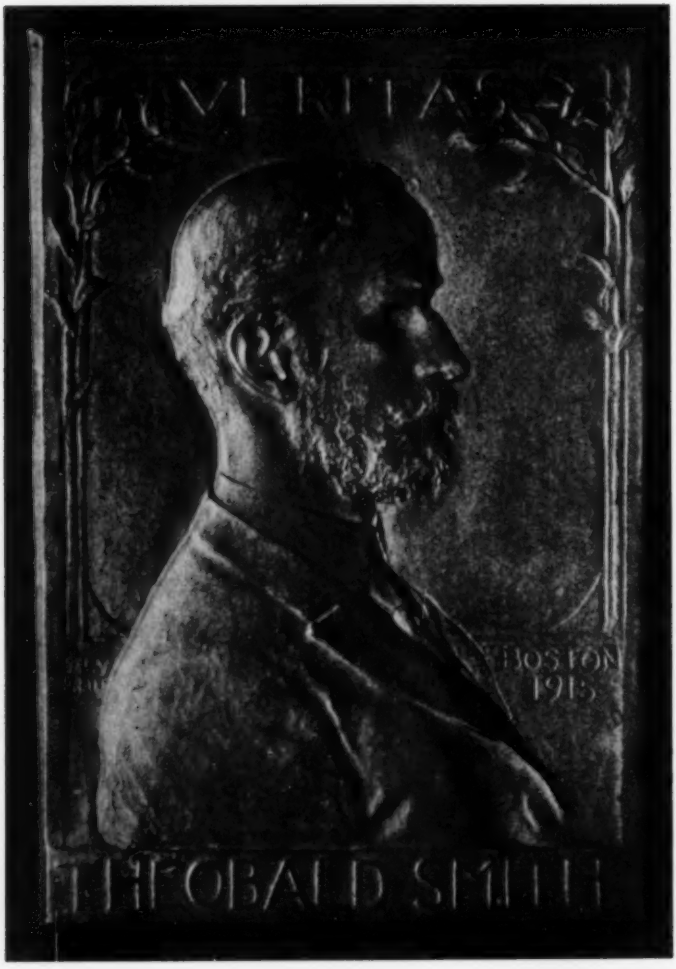
BOSTON
1915
THEOBALD SMITH

Theobald Smith[1]
1859–1934

Theobald Smith was one of that small group of pioneer bacteriologists in America who grasped the unique opportunity of developing a science which has revolutionized medicine and some branches of industry. He was an American bacteriologist who did not go to Europe for study under the early masters of his subject but to a remarkable degree was self-taught, developing his own methods. Beginning work in the eighties and early nineties of the last century, the period of the most rapid discovery of pathogenic microörganisms, he has said that during this exciting period it was difficult to wait for the daily newspaper to learn what pathogenic organism had been discovered. He was, however, no mere microbe hunter. To him the discovery of an etiological agent was only the introduction to the larger problems of the interrelations of host and parasite. The subjects of his many publications include: the method of transmission of infectious agents from one individual host to another, as in the insect transmission of the parasite of Texas fever, the rôle of Heterakis in the production of enterohepatitis of turkeys; the differentiation of closely related organisms, as the varieties of tuberculosis bacilli, the Brucella, the paratyphoid bacilli, the streptococci and the anaerobes; simple and ingenius methods of cultivating and differentiating bacteria, as the use of the fermentation tube, the use of bits of fresh unheated animal tissue in media and the differential value of sugar fermentation; comprehensive studies of infections natural to animals; the production of immunity by heat killed cultures; the transmission of antibodies in utero and through the colostrum. Although his attention was often focused

[1] The picture reproduced herewith is of the bronze plaque by Bela Pratt which is mounted in the hall of the Department of Comparative Pathology at Harvard Medical School.

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upon the investigation of infectious diseases of animals, his excursions into pure bacteriology, parasitology or immunology were more frequent than those into histological pathology. Of his 250 publications no less than 75 may be said to have been in the field of pure bacteriology. It is interesting to note that 14 papers appearing throughout the years 1886 to 1932 were concerned with the variability of bacteria but that his interpretation of these results was always conservative. He knew how to take advantage of the by-products of research without losing sight of the main problem and was always alert for incidental discoveries which in many instances were the first observation of these phenomena, such as the existence of flagellar and somatic agglutinins, the immunizing effect of toxin-antitoxin mixtures, the Theobald Smith anaphylactic phenomenon and scurvy in guinea pigs. His published papers are well distributed in the fields of bacteriology, parasitology, immunology, public health and pathology. The synthesis of his discoveries and observations is given in the Vanuxum Lectures for 1934 on Parasitism and Disease.

Educated as a physician it is difficult to say whether Dr. Smith's devotion to comparative pathology was more the result of choice or opportunity but he soon recognized that disease is a natural biological phenomenon common to all living things, that the fundamental phenomena are the same whether in man or animals whereas the latter often afford the best opportunity for observation and experiment. For this reason he preferred the study of infections natural to animals rather than the study of animals inoculated with human pathogens. By way of illustration he has said that more could be learned about typhoid by studying paratyphoid in animals than by studying animals inoculated with typhoid bacilli. His appreciation of the importance of animal industry, the economic dependence of man upon animals, his interest in the diseases of animals and in those infections common to animals and man caused him to give equally sympathetic consultation to the animal husbandryman, veterinarian and physician. He served successively in the Bureau of Animal Industry of the U. S. Department of Agriculture, as Professor

of Comparative Pathology at Harvard University and as Director of the Department of Animal Pathology of The Rockefeller Institute for Medical Research.

Dr. Smith's characteristics of mind and habits of work inspired in those who worked with him the highest confidence and respect. He was meticulously careful and thorough. Details were too important to be slighted or delegated to others. He kept careful notes on all that he observed. A series of notes started, filed and added to as opportunity permitted was never complete but always a growing piece of research. When a report of work was published it often represented observations made over a long period of years; it was mature, carefully written, moderately stated and as nearly as possible correct in every detail. This is his reputation. To him waste of effort or material, even in the midst of plenty, was inexcusable, but effort and material spent in thorough work was never wasted regardless of the result obtained. He did not exert undue pressure upon those working under him. With verbal criticism he was always moderate. He rarely offered praise. To be left alone by him was a compliment and made one feel that much was expected. As a writer, lecturer and teacher he presented clearly what was essential and nothing superfluous. By example and with few words he exerted a subtle influence that left its mark on those who worked with him.

Nothing that might be said could more eloquently describe the scope, originality and permanent value of Theobald Smith's contributions to science than a list of his publications. To read them is to be impressed by astute observations which only now seem new and there are others which must be rediscovered to be appreciated. Their author would ask no other recognition or reward.

J. Howard Brown.

A SEROLOGICAL STUDY OF HAEMOLYTIC STREPTOCOCCI

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We have endeavoured to arrange in groups a large number of strains of haemolytic streptococci from human beings and a much smaller number from animals. The results of macroscopic agglutinin-absorption tests carried out with 6 and in many instances 9 different type sera, absorbed respectively with 420 strains, are here presented. These results are correlated with group precipitin tests and certain well known biochemical reactions.

Many attempts to classify haemolytic streptococci into definite serological groups by means of agglutination tests have met with such difficulties as spontaneous clumping of organisms, cross agglutination with different types of immune sera, and partial absorption of agglutinins. To avoid spontaneous agglutination, Dochez, Avery and Lancefield (1919), Bliss (1922), Durand and Sédallian (1925) and Rosner (1928) grew antigens in a salt-free medium containing phosphate buffer; Tunnicliff (1922) used ascitic broth plus glucose; Gordon (1921) used trypagar plus ascitic fluid; Shibley (1924) allowed the organisms to grow at room temperature; Smith (1926) suspended washed organisms in 0.001 N/1 NaOH and diluted the immune serum with M/320 NaCl solution; James (1926) grew organisms first in salt-free meat infusion broth, then transplanted them to tryptic digest broth; Noble (1927) used serum and antigen in a more concentrated form than is usual, reading the results after the mixture was shaken for 2 minutes; Stevens and Dochez (1926), Spicer (1930) and Williams and Gurley (1932) added strips of sterile potato to phosphate-buffered broth; McLachan and Mackie (1928) used phosphate-

buffered broth plus glucose; Gunn and Griffith (1928) and Allison (1931) used a slide agglutination test observed microscopically; Mueller and Klise (1932) diluted immune serum with physiological saline containing 2 per cent normal horse serum. To avoid cross agglutination, agglutinin-absorption tests have been chiefly used. Griffith (1927) and Spicer (1931) reported satisfactory results with a slide agglutinin-absorption test. Andrewes and Christie (1932) by means of a series of graded absorptions concluded that very few haemolytic streptococci are serologically identical with any one of the four scarlet fever types of Griffith or with the Dochez N. Y. 5 strain.

Lancefield (1933), using a precipitin test, classified into 5 groups all except 2 of 106 strains of haemolytic streptococcus isolated from man, animals, milk and cheese; later work (1934) showed that all except 3 of 21 strains of Group B (so-called bovine strains) could be further classified into 3 sharply defined and specific types.

METHODS

1. Acid production. After incubation for 48 hours the final acidity of a 1 per cent glucose broth culture was determined colorimetrically by the method of Avery and Cullen (1919). As a rule readings were made in duplicate with methyl red and brom-cresol-green as indicators.

2. Hydrolysis of sodium hippurate. The medium, made according to the method of Hardenburgh (1930), was dispensed into 5 cc. quantities and seeded with a loopful of culture from a solid medium. If hydrolysis of sodium hippurate occurred, the addition of 0.5 cc. of a 7 per cent aqueous solution of ferric chloride to 2 cc. of a 72-hour culture caused a precipitate of benzoate to form.

3. Fermentation reactions. The medium consisted of sugar-free meat infusion broth containing 1 per cent Difco peptone, 0.5 per cent NaCl, 1 per cent normal horse serum,1 per cent Andrade's indicator and 1 per cent respectively of the following carbohydrates: lactose, sucrose, salicin, mannitol, raffinose, inulin, sorbitol and trehalose. Carbohydrate media were seeded with 1 loopful of fresh 24-hour serum broth culture of the strain under test and, unless fermentation took place earlier, incubated for

7 days. The purity of the culture in carbohydrate media was verified by streaking on blood agar and by microscopic examination of a film stained with Gram's stain. Whenever a culture did not conform to the pyogenes group of Holman (1916), the carbohydrate test was repeated at least 3 times.

4. Reduction of methylene blue. The method of Avery (1929) was used as follows: to skimmed milk sterilized by heating at 250°F. for one-half hour was added a 1 per cent aqueous solution of methylene blue, so that the final concentration of dye was 1:5000 (2 cc. solution of methylene blue: 100 cc. milk). Sterile milk, with and without dye, was dispensed into 5 cc. quantities and seeded with 0.1 cc. fresh 18-hour serum broth culture of the strain to be tested. Observations were made after incubation at 37°C. for 24 hours, 3 and 7 days, respectively. The methylene blue was seldom reduced after 24 hours.

5. Growth on blood agar containing bile. A slight modification of the method of Belenky and Popowa (1929) was used as follows: 10 per cent sheep's blood agar plates containing 10 and 40 per cent sterile ox bile respectively were streaked with a loopful of fresh 18-hour serum broth culture of streptococcus. In addition, a control without bile was incorporated in the test. Readings of the amount of growth as contrasted with the control plate were made after incubation at 37°C. for 48 hours.

6. Extracts for precipitin test. Using a slight modification of the method of Lancefield (1933), and of Edwards (1934), the bacterial sediment from 100 cc. of an 18-hour serum-broth culture was washed once with physiological saline and then taken up in 2 cc. of saline, containing sufficient normal hydrochloric acid to give a final concentration of N/20 HCl. Congo red paper was used as indicator and the acidity of the suspension was always made such as to turn the paper blue. Tubes containing the acidulated bacterial sediments were heated for 10 minutes in boiling water, then cooled and neutralized with N/1 NaOH. Brom-thymol-blue, used as indicator, was added directly to the cooled suspension. After centrifugalization the supernatant of the neutralized culture was used as the extract in the precipitin test.

7. Precipitin tests. In the majority of these tests, to avoid

slight cross-precipitation, the immune serum was absorbed for 2 hours at 37°C. with an equal volume of heterologous extract; after standing overnight at room temperature the supernatant of the centrifugalized absorbed serum was used in the precipitin tests. In later work satisfactory tests were made with unabsorbed serum provided that the bacterial growth was washed once with saline before extraction with acid. To 0.4, 0.1, and 0.025 cc. of extract respectively, made up to a final volume of 0.4 cc. with saline, was added a constant volume of serum (0.2 cc. with absorbed, and 0.1 cc. with unabsorbed, serum). As a control, to 0.4 cc. of each extract was added 0.1 cc. normal rabbit serum. After standing for 10 minutes at room temperature, the tubes were observed for the presence of a ring at the junction of the serum and extract, shaken and subsequently incubated in a water bath at 37°C. for 2 hours; final readings were made after the tubes had stood overnight in the refrigerator. Usually a ring was noted in one or more tubes of each set where precipitation was later found. A test was interpreted as positive if precipitation occurred in any one of the three tubes.

8. Immune sera. Rabbits were immunized by intravenous injection of formalin-killed cultures prepared according to the method of Mueller and Klise (1932). From 4 to 6 weeks were usually adequate for the production of antiserum having an agglutinating titre of at least 1:1280, or a suitable precipitating titre. To the immune blood serum was added, as a preservative, 1 part of merthiolate to 10,000 parts of serum.

9. Bacterial antigens. Antigens were prepared from cultures grown for 18 hours at room temperature in meat infusion broth containing 1 per cent Difco peptone and 0.5 per cent NaCl at pH 7.6, the broth having been sterilised by filtration through a Seitz filter. If the bacterial growth did not yield an even suspension after 3 or 4 daily transplants, the supernatant of the centrifugalized culture was used for seeding fresh broth, repeating the transplants of supernantants until a satisfactory result was obtained. Fresh living 18-hour broth cultures were used in the tests. The density (McFarland scale Tube II) of such cultures was satisfactory for use as antigens.

10. Absorption of agglutinins. Cultures for absorption were grown for 48 hours at 37°C. in beef infusion broth containing 1 per cent Difco peptone and 0.5 per cent NaCl at pH 7.6, sterilized by heating for one-half hour at 250°F. After adding 1 per cent sterile horse serum the broth was seeded with a loopful of culture from a solid medium. Sediment from 70 cc. of a 48-hour serum broth culture taken up in 0.9 cc. saline and added to 0.1 cc. of serum diluted 1:10 sufficed to remove all of the agglutinins from the autologous serum. Absorption proceeded for 3 hours in a water bath at 37°C., then at room temperature until the following day, when the organisms were removed from the serum by centrifugalization.

11. Agglutinin-absorption tests. In the majority of tests 6 different serial dilutions of each absorbed serum were used, from 1:100 to 1:3200. To 0.4 cc. of each serum dilution was added 0.4 cc. autologous antigen, the mixture being shaken, then placed in a water bath at 56°C. for three to four hours. In all except the very earliest tests, readings were made immediately after removal of tubes from the water bath as well as on the following morning after the tubes had stood overnight in the refrigerator. The overnight readings were used since the readings were more easily made and the titre was higher. The titre of the serum was determined from the highest dilution in which any degree of agglutination was definitely present. Controls of antigen plus saline and unabsorbed serum respectively were incorporated into each test. The titre of serum absorbed by an heterologous strain was usually slightly lower than that of the unabsorbed serum both of which had been subjected to the same temperature throughout the whole test. Storing a serum in the refrigerator for a period of 2 years or longer caused the agglutinating power to be decreased.

RESULTS

Acid production, hydrolysis of sodium hippurate, fermentation of trehalose and sorbitol

The first five columns of table 1 show the origin of the cultures and their grouping according to biochemical reactions already

TABLE 1

Summary of biochemical and precipitin reactions

	FINAL pH	HYDROLYSIS SODIUM HIPPURATE	TREHALOSE	SORBITOL	NUMBER OF CULTURES	METHYLENE BLUE NOT REDUCED	INHIBITION IN BILE‡ BLOOD AGAR		PRECIPITIN TESTS ANTISERA FOR GROUP			
							10 per cent	40 per cent	A	B	C	G
Group A (346 strains):												
Scarlet fever	5.0–5.2	–	+	–	77	60	38	72	77	0	x	x
Dick I, II, III, IV, B1, 2, 3, 4, 5, 6; N. Y. 5	5.0–5.2	–	+	–	11	11	3	10	11	0	0	x
Puerperal sepsis	5.0–5.2	–	+	–	47	35	7	39	44	0	2	x
Erysipelas	5.0–5.2	–	+	–	13	7	4	9	10	0	3	x
Miscellaneous (sick)	5.0–5.2	–	+	–	60	43	24	55	60	0	x	x
Miscellaneous (well)	5.0–5.2	–	+	–	8	4	4	7	8	0	x	x
Institution O*	5.0–5.2	–	+	–	29	7	3	16	28	0	1	x
Institution B	5.0	–	+	–	49	21	32	47	49	0	x	x
Horse-strangles	5.0	–	+	–	1	0	0	0	0	0	1	x
K. L. epidemic (1930):†												
Cases	5.0	–	+	–	20	17	9	19	20	0	x	x
Contact	5.0	–	+	–	1	0	0	1	1	0	0	x
Milk handlers	5.0	–	+	–	7	6	0	6	6	0	1	x
School group	5.0–5.2	–	+	–	21	11	6	16	20	0	1	x
Cows	5.0	–	+	–	2	0	1	2	2	0	0	x
Per cent						64.2	39.0	86.4	97.1	0	2.6	x

Group B (59 strains):												
K. L. milk handlers	4.4	+	+	−	2	0	0	0	0	2	0	x
Cows	4.4	+	+	−	2	0	1	1	0	2	0	x
Scarlet fever convalescents	4.4–4.6	+	+	−	2	0	1	2	0	1	0	x
Arthritis—prostatic secretion	4.6	+	+	−	1	0	0	0	0	1	0	x
Laryngitis	4.6	+	+	−	1	0	0	0	0	1	0	x
Rabbit's brain	4.4	+	+	−	1	0	0	0	0	1	0	x
Institution B	4.4–4.6	+	+	−	50	16	1	0	0	50	0	x
Per cent						27.1	5.1	5.1	0	98.3	0	x
Group C (10 strains):												
Rabbits, horses, guinea pigs	5.0–5.2	−	−	+	10	10	3	9	0	0	10	x
Per cent						100.0	30.0	90.0	0	0	100.0	x
Group G (3 strains):												
Normal throat	4.4	−	+	−	1	0	0	0	0	0	0	1
Tonsillitis	4.6	−	+	−	1	1	0	0	0	0	0	1
K. L. school child	4.4	−	+	−	1	0	0	0	0	0	0	1
Proportion of group F.						1/3	0	0	0	0	0	3/3

* Institutions O and B, cultures from throats of children exposed to scarlet fever.
† K. L., Kirkland Lake septic sore throat epidemic.
‡ Numbers represent the number of cultures having growth totally or partially inhibited by bile.
x indicates test not performed.

reported by the author (1934). The bacteriological investigation of the Kirkland Lake cultures has been reported by McNabb, Fraser and Fraser (1931).

For purposes of uniformity the groups (A, B and C) are designated in accordance with the classification of Lancefield (1933).

We feel that group A made up of 346 strains obtained chiefly from man, may be regarded as representative of the majority of hemolytic streptococci isolated from human beings. Two strains from the milk of cows incriminated in an epidemic of septic sore throat and 1 strain[1] from a horse suffering from strangles, fit into this group. Group B is very interesting, but since 56 of the 59 strains were isolated from human beings, it cannot be considered as necessarily representative of the usual bovine type of streptococcus isolated from cows. Fifty of these strains were from the throats of children in Institution B where raw milk was used, 2 from the throats of milk handlers, 2 from convalescent cases of scarlet fever and 2 from people suffering respectively from arthritis and laryngitis; from animal sources were 1 from the brain of a rabbit inoculated intracerebrally with vaccine virus from a calf and 2 from cow's milk. Group C is small, containing only 10 strains of sorbitol-fermenters isolated from rabbits, horses and guinea pigs. The fourth group which did not correspond to any of Lancefield's (1933) was shown later to be identical with her group G, reported in 1935.[2]

Reduction of methylene blue. No distinct grouping of cultures was possible by means of this test since 64.2 per cent of 346 cultures of group A (human) and 27.1 per cent of 59 cultures of group B (bovine) did not reduce the dye (see table 1). Our findings agree with those of Brown (1920) who was unable to differentiate streptococci of human and bovine origin by the reduction of methylene blue. Sherman and Albus (1918) and Seelemann and Hadenfeldt (1930) reported that streptococci of bovine origin which hydrolyzed sodium hippurate also reduced methylene blue, whereas those of human or of bovine origin which did not hydrolyze sodium hippurate did not reduce the dye. Klimmer, Haupt

[1] Biochemically group A, but by precipitin test, group C.

[2] See Lancefield, R. C., and Hare, R., J. Exp. Med., 1935, *61*, 335.

and Roots (1928) reported that not all strains of streptococci isolated from milk both hydrolyze sodium hippurate and reduce methylene blue.

Bile blood agar. This test gave no sharp distinction between cultures of different groups, 10 per cent bile inhibiting partially or totally the growth of 39.0 per cent of human strains and of 5.1 per cent of bovine strains; with 40 per cent bile the corresponding figures were 86.4 and 5.1 per cent (see table 1). Seelemann and Hadenfeldt (1930) found that streptococci which hydrolyze sodium hippurate were not inhibited by 10 or by 40 per cent ox bile, whereas non-hippurate hydrolyzers grew only to a slight extent on a medium containing 10 per cent and not at all in the presence of 40 per cent bile. Bonciu (1929) reported that only 12 of 160 strains from scarlet fever cases were inhibited by the presence of 10 per cent ox bile.

It is conceivable that the information gained from the methylene blue and the bile blood agar tests may assist in the classification of streptococci according to their source, but in our hands this information alone did not give a sharp distinction between bovine and human strains.

Precipitin tests. Precipitating sera were prepared for one or more strains of groups A, B, C and G respectively. Each extract was tested with anti-human (group A) and anti-bovine (group B) absorbed serum. With unabsorbed anti-C serum, tests were repeated with 102 different extracts, 24 of which had not been classified into either group A or B. The 5 extracts still unclassified were retested with anti-G serum and the 3 raffinose-fermenters fell into group G. The biochemical grouping agrees remarkably well with the grouping as determined by precipitin tests (table 1). No strain gave a positive reaction with more than one group serum. Since 2 strains did not give a positive reaction with any serum and since 9 strains of group A gave a positive reaction with group C anti-serum, a study of the carbohydrate reactions was made in the hope of finding some explanation for these exceptions. Neither of the 2 strains giving a negative precipitin reaction belonged to the pyogenes group, 1 being a salicin non-fermenter from a case of puerperal sepsis and 1 a sucrose non-fermenter

from a convalescent case of scarlet fever. Two of the 8 human strains giving a precipitin reaction with anti-C serum were of the pyogenes group, 5 were lactose non-fermenters, 1 a salicin non-fermenter and 1 both a salicin and lactose non-fermenter. In group A there were 319 pyogenes strains, plus 12 mannitol fermenters and 5 non-lactose fermenters. Of the group B strains, 15 were pyogenes, 42 salicin non-fermenters and 2 lactose non-fermenters. The large proportion of salicin non-fermenters in our group B should not be accepted as typical of bovine strains because these were all isolated from the throats of children in Institution B.

Thus the carbohydrate reactions offer no explanation for the serological relationship between the 9 strains[3] of group A and the 10 animal strains of group C.

Agglutinin-absorption tests. Anti-sera were prepared for the following strains of haemolytic streptococci: B1, B2, B3, B4, B5, and B6 isolated from cases of scarlet fever by Dr. J. H. Mueller and received through the courtesy of Dr. Alice Evans; X40 from the 1912 Chicago epidemic and C108 from the 1917 Boston epidemic of septic sore throat received from Dr. J. H. Brown; S. F. type I and S. F. sub-type I from Dr. Anna Williams; KL55 from a case and KL56 from a milk-handler in the 1930 Kirkland Lake epidemic of septic sore throat; Dochez (N. Y. 5) a strain of scarlatinal origin, carried in this laboratory for the past eight years. Preliminary agglutinin-absorption tests showed that strains B3, S. F. sub-type I and KL56 were serologically identical so that only the latter anti-serum was used. Since anti-serum B4 was shown to possess mainly group agglutinins in 128 different tests, and since strain B2 was capable of absorbing nearly all of the B4 agglutinins, this serum was not used for typing purposes. Although partial absorption of agglutinins from Dochez anti-serum occurred with two-thirds of the strains of type B1, in only 1 case was there actual doubt as to the type and the third test showed this strain to belong to type B1. According to Mueller and Klise (1933), the four types of Griffith are serologically identical with

[3]Group A by biochemical tests.

the types numbered with corresponding numbers. Andrewes and Christie (1932) have reported a close serological relation between Griffith's scarlet fever types II and IV and some relation between Griffith's type I and the Dochez N. Y. 5 strain. In this case, our results with anti-sera B4 and Dochez confirm the findings of Andrewes and Christie.

For the most part results were not difficult to interpret. Out of many retests, only 6 were not confirmed. In 3 instances the original test and in 2 cases the second test was in error. With one strain 3 tests placed it in both types B1 and Dochez, but the fourth test placed it in type B1 only.

Table 2 shows the results with 420 strains. The members of groups B, C and G did not fall into any of the types tested, and they are not included in any totals given in table 3. Cultures from the throats of children in Institutions B and O were taken after the occurrence of several cases of scarlet fever in these institutions. Since it is difficult to say whether or not these cultures should be considered as scarlet fever contacts, we have not included them with the other contact strains, nor in the summary shown in table 3.

Grouping the cultures from cases and contacts of scarlet fever, we have 50.6 per cent of 83 strains belonging to 5 types (B1, B2, KL56, B5, and B6), whereas 36.3 per cent of 187 human strains of non-scarlatinal origin belong to 8 types (B1, B2, KL56, B5, B6, KL55, X40 and C108). Omitting the Kirkland Lake cultures from the latter group, 18.3 per cent of 131 cultures of non-scarlatinal origin belong to 8 types. None of our strains fitted into the Dochez type, which finding agrees with that of James (1926) and of Smith (1927). The most prevalent type in this district appears to be KL56 or the B3 group of Mueller and Klise.

Table 3 shows a comparison of results obtained with agglutination tests by different experimenters. The percentage of our cultures fitting into distinct types was lower than that of most workers. This fact may be in part due to testing cultures collected over a period of 10 years, and again we may not have had anti-sera which would classify the majority of strains prevalent in this district.

The most valuable information from the agglutination tests was found from an analysis of results obtained from the Kirkland

TABLE 2

Agglutinin-absorption tests

STRAINS	ANTI-SERA FOR STRAINS									NOT TYPED
	B1	B2	KL55 (B3)	B5	B6	KL55	Dochez	X40	C108	
Group A:										
Scarlet fever:										
Cases	13	5	13	0	1	0	0	0	0	33
Contacts	0	0	5	1	3	0	0	0	0	2
Carriers	1	0	0	0	0	0	0	0	0	2
Dick I, II, III, IV	0	0	0	0	0	0	0	0	0	4
Institution B	0	0	22	4	0	0	x	x	x	23
Institution O:										
Dick positive	1	0	1	0	0	0	0	x	x	11
Dick negative	0	0	1	0	0	0	0	x	x	15
Septic sore throat, Kirkland Lake (1930):										
Cases	x	x	2	x	x	17	0	x	x	1
Contacts	x	x	1	x	x	x	0	x	x	0
Diagnosis uncertain	x	x	1	x	x	x	0	x	x	0
School group	0	0	7	3	0	5	0	x	x	7
Milk handlers	0	0	3	0	0	3	0	x	x	4
Milk	0	0	0	0	0	2	0	0	0	0
Lee #76, #29A (1930)	0	0	0	0	0	0	0	0	0	2
Erysipelas	1	0	0	0	0	0	0	1	0	11
Puerperal sepsis:										
Blood	0	0	0	0	0	0	0	x	x	10
Cervix	1	0	0	1	0	0	0	x	x	26
Abscess	0	0	0	0	0	0	0	x	x	2
Contacts (throats)	0	0	0	0	0	0	0	x	x	5
Miscellaneous streptococcal infections	4	3	1	5	1	2	0	3	0	39
Normal throats	0	0	0	0	0	0	0	0	1	12
Total	21	8	57	14	5	29	0	4	1	209
Group B	0	0	0	0	0	0	x	x	x	59
Group C	0	0	0	0	0	0	0	x	x	10
Group G	0	0	0	0	0	0	0	x	x	3

x, agglutination-absorption test not done.

Lake epidemic of septic sore throat. From one dairy which supplied milk to 84 per cent of the families in which cases occurred,

group A strains of haemolytic streptococci were isolated from the milk of 2 cows and from the throats of 4 of the 6 milk-handlers, all of whom had no history of recent illness. (It is true that about 2 months before the epidemic 2 cases of scarlet fever had occurred in the family of the dairy owner, a third member developing a cold with a "running ear".) These strains isolated belonged

TABLE 3

Comparison of results obtained with agglutination tests by different experimenters

EXPERIMENTER	SCARLATINAL GROUP			NON-SCARLATINAL GROUP		
	Number tested	Per cent typed	Number of types	Number tested	Per cent typed	Number of types
Direct agglutination						
Dochez, Avery and Lancefield (1919)				125	68.0	4
Bliss (1920)	25	80.0	1	17	18.2	1
Tunnicliff (1922)	36	91.7	1	39	17.9	1
Gunn and Griffith (1928)	91	66.0	4			
Rosner (1928)	44	82.0	4	33	18.0	4
Allison (1931)	600	63.0	4	396	2.0	4
Mueller and Klise (1933)	225	63.1	6			
Agglutinin-absorption tests						
Gordon (1921)	18	88.9	1	114	99.1	3
Durand and Sédallian (1925)				130	64.6	6
Smith (1927)	210	98.0	7	72	86.1	9
James (1926)	53	56.6	3	43	23.3	3
Griffith (1927)	222	70.2	4			
McLachlan and MacKie (1928)	101	76.2	4	30	46.6	4
Plummer	83	50.6	5	131*	18.3	8
				187†	36.3	8

* Cultures excluding those from Kirkland Lake epidemic of septic sore throat.
† Cultures including those from Kirkland Lake epidemic of septic sore throat.

to either type KL55 or KL56, one person harbouring each type at a different time. Two other cows and two other milk-handlers yielded haemolytic streptococci belonging to group B. In four instances, 2 cases of septic sore throat occurred in the same family, and in two of these homes one patient carried type KL55 and the other type KL56. McKay and Hardman (1931) have reported the occurrence of several cases of scarlet fever in Kirk-

land Lake just previous to the septic sore throat epidemic, and cultures from the nose and from the throat of one case of scarlet fever at the time of the epidemic each belonged to type KL56. From the septic sore throat cases and contacts, 20 out of 21 cultures belonged to one or other of types KL55 and KL56. The one remaining culture which was untyped was from a public health nurse, whose case was atypical manifesting only slight illness. From the school group 68.2 per cent of 22 cultures belonged respectively to types KL55, KL56 and B5. The school children did not furnish a normal control group since several of them had sore throats or a history of sore throats or colds in the family. The cultures of type KL55 were from 2 children with sore throats, 1 with a "digestive upset," and 2 without history of recent illness; those of type KL 56 were from 2 children with sore throats, 2 with a history of sore throat or of scarlet fever in the family and 3 without any history of recent illness; the 3 cultures of type B5 were from children without history of recent illness. Of the positive throat cultures from children using milk from the dairy implicated, 5 were of type KL55, 1 of KL56 and 1 untyped. The evidence lends strong support to the theory that we are dealing here primarily with a milk-borne epidemic due to type KL55, and secondarily with a strain which was apparently associated with the previous scarlet fever epidemic.

CONCLUSIONS

Of any single test used, the precipitin reaction gives the most information concerning the group to which a culture belongs. Only 11 (2.6 per cent) of 418 strains were not placed in the group to which they belonged by biochemical reactions (final pH, hydrolysis of sodium hippurate, fermentation of trehalose and sorbitol). Nine of these 11 strains which would have been expected to fall into group A, fell into group C, the other 2 strains not falling into any group.

The agglutinin-absorption test is far too delicate and specific to give much information concerning the source of cultures because so few organisms fall into the same type. If, however, cultures are found to fit into a type, their close relationship is definitely established. As an example, in our study of the Kirk-

land Lake group of cultures, we concluded that 20 out of 21 cases of septic sore throat were caused by one or other of two types of streptococcus, one type being found in the milk used, and both types in the throats of the milk-handlers at the dairy; no other test could have given us such definite information.

Fifty-six of the 59 strains belonging to group B were isolated from human beings, most of whom were not ill. These strains have all of the characteristics associated with haemolytic streptococci from bovine sources. Since this report was written another group-B strain has been isolated from the throat of a well person in an Institution where several cases of scarlet fever occurred. We suggest that the term "bovine" should refer to the characteristics rather than to the source of the strain.

My gratitude is extended to Dr. Frieda Fraser for the isolation of most of the cultures and for valuable suggestions, and to Misses J. Cooke and A. Carson for technical assistance in carrying out the different tests. To Dr. R. C. Lancefield I am greatly indebted for her assistance in identifying our fourth group with her group G.

SUMMARY

1. By means of agglutinin-absorption tests, 50.6 per cent of 83 strains of haemloytic streptococci from cases and contacts of scarlet fever were classified into 5 types; similarly 18.3 per cent of 131 strains of non-scarlatinal origin were placed in 8 types.

Of 21 cultures from cases and contacts in the Kirkland Lake septic sore throat epidemic, 20 belonged to one or other of 2 types; milk from a dairy which supplied 84 per cent of the families having cases yielded one of these types and cultures from the throats of milk-handlers yielded both types.

2. By means of precipitin tests, all but 2 out of 418 cultures were classified in one or other of 4 groups. This classification agrees remarkably well with one based upon biochemical reactions.

3. Reduction of methylene blue gave no distinct classification of organisms, although human strains showed fewer reductions of the dye than did the bovine strains.

4. The presence of ox-bile in blood agar inhibits the growth of

human strains more than that of bovine strains, but no clear-cut division could be thus made.

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THE INHIBITION OF GROWTH OF BACILLUS SUBTILIS ON A MODIFIED EXTRACT AGAR BY X-RADIATION OF THE MEDIUM

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While investigating a problem concerned with the killing by X-rays of *Bacillus subtilis* spores on the surface of extract agar, it was observed that irradiation of the medium alone caused a marked diminution in the number of colonies which would develop, as compared to the growth on a control non-irradiated medium. A modified medium, on which *B. subtilis* grew satisfactorily, could be so altered by the action of X-rays that it no longer permitted the germination of spores and the subsequent multiplication of vegetative cells into macroscopically visible colonies.

A review of the literature has shown that few investigators have been concerned with the action of X-rays on the medium alone. Brown, Luck, Sheets and Taylor (1933) found no evidence that X-radiation of nutrient broth or yeast autolysate for nine to thirty-five minutes (2.5×10^3 R-units per second) produced substances of appreciable toxicity to *Pseudomonas fluorescens* or *Escherichia coli*. Several workers, however, have investigated the effect of ultra-violet light and have arrived at conclusions which are of interest in comparison with the results of this research. Woodrow, Bailey and Fulmer (1927) irradiated a water solution of sucrose, NH_4Cl, K_2HPO_4 and $CaCl_2$ with ultra-violet light and found that the rate of growth of *Saccharomyces cerevisiae* was decreased as compared to the growth in a similar non-irradiated solution. This decrease, they thought, resulted from the formation of a non-volatile, toxic material by the action

of ultra-violet light on sucrose. Proks (1933) obtained inhibition of growth by ultra-violet irradiation of gelatin and agar media. The following report is concerned with an attempt to determine the mechanism of the inhibition of growth of *B. subtilis* on a modified extract agar by the X-radiation of the medium.

The X-rays were supplied by a gas X-ray tube as previously described by Kersten (1934). The arrangement of the samples in relation to the window of the X-ray tube is shown in figure 1. A copper target was used for all of this work. A photograph taken with an oscillating crystal spectrograph showed that the

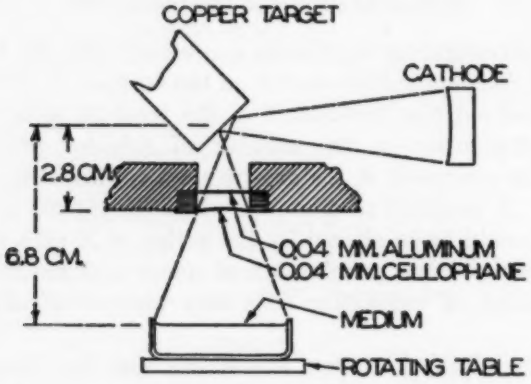

FIG. 1. SHOWING THE RELATIVE POSITIONS OF THE TARGET, WINDOW, AND MEDIUM

most intense part of the radiation consisted of the K_α (1.54 Å) and K_β (1.38 Å) lines of copper. The voltages mentioned later were measured by a spark gap placed across the tube and therefore represented peak voltages.

It was thought advisable, for reasons to be considered later, to use such an area of medium that the entire surface could be irradiated and not just a portion of a larger surface as had been done by some previous workers (Burger, 1931; Lacassagne, 1928) when irradiating bacteria on the surface of solid media. The X-ray beam used was 19 mm. in diameter at the window, diverging to approximately 5 cm. at 4 cm. from the window. We have

used two types of containers: a micro-culture slide in which there was a depression 15 mm. in diameter and 3 mm. deep, for the irradiation of materials as near to the window as possible, and petri dishes 4.0 to 4.8 cm. in diameter and 1.0 to 1.3 cm. deep, for irradiation 4 cm. from the window.

The suspension for inoculation was always prepared in the following manner. *B. subtilis* was grown on a standard extract agar slant for five days at 30°C. This growth was then washed off with a 0.9 per cent sodium chloride solution and diluted each time to a density as nearly constant as possible. No counts were made. The suspension was then held in a water bath at 70°C. for one hour. It was felt that such an inoculum when reproduced from day to day was more nearly constant than a mixture of spores and vegetative cells or a suspension of a younger culture. Irradiated and control plates were always inoculated with a sterile cotton swab moistened with the suspension. Subsequent incubation was at 30°C.

The term "control," in each experiment, refers to a final medium prepared exactly the same as the experimental medium except that irradiation was omitted. By "inhibition of growth," the authors mean the inability of the spores to produce macroscopically visible colonies. The spores may germinate and yet no visible growth appear. This latter phenomenon was not investigated. Sub-cultures onto control media from the surfaces of irradiated media at varying periods following inoculation produced macroscopically visible growth, thus indicating that the irradiated media may "inhibit growth" without actually killing the spores.

I. ACTION OF X-RAYS ON THE ENTIRE MEDIUM

When regular extract agar (0.3 per cent beef extract, 0.5 per cent NaCl, 1.0 per cent Bacto-peptone and 2.0 per cent agar) was irradiated, at voltages of 20 to 38 kv., with a current of 10 m.a. for thirty to sixty minutes, as near the window as possible (3.0 cm. from the focal spot) and later inoculated, there invariably resulted a diminished growth on the irradiated medium as compared with the control. It was found possible to reduce the

concentration of both the beef extract and the peptone to one-tenth of the above values or to omit peptone completely and reduce the concentration of the beef extract by one-half and still obtain a medium satisfactory for the growth of *B. subtilis*. When such media were irradiated at 38 kv., 10 m.a., 3.0 cm. from the focal spot for forty-five minutes and subsequently inoculated, no colonies of *B. subtilis* appeared after twenty-four hours incubation. The final composition of the medium in each of the following experiments was 0.15 gram beef extract, 0.5 gram NaCl and 2.0 grams agar per 100 ml. except where specifically stated otherwise.

Experiment 1. Four sterile slides were filled aseptically with medium. One was irradiated at 38 kv., 10 m.a., 3.0 cm. from the focal spot for thirty minutes; the second for fifteen minutes; the third for eight minutes; and the fourth not at all. Immediately after irradiation, the slides were inoculated and incubated for seventy-two hours. One colony appeared on no. 1, one on no. 2, six on no. 3, and normal confluent growth on no. 4.

A comparison of the amount of time necessary to inhibit growth upon a larger quantity of this medium is obtained by the following experiment which is typical of the general technique employed for irradiating 10 to 15 ml. quantities of media.

Experiment 2. Of the medium, 10 ml. were placed in a petri dish and sterilized. After solidifying, the medium, continually rotating at 1 R.P.M., was irradiated at 38 kv., 10 m.a., for two hours, the top of the petri dish having been removed. Immediately after irradiation, it was inoculated and incubated for forty-eight hours. No colonies of *B. subtilis* developed, while the control plate showed normal confluent growth.

II. ACTION OF X-RAYS ON THE INDIVIDUAL CONSTITUENTS OF THE MEDIUM

Following this definite proof of the ability of soft X-rays to inhibit the growth of *B. subtilis* by irradiation of the medium previous to inoculation, the effect of the irradiation on the individual constituents of the medium was studied.

The work of Taylor, Thomas, and Brown (1933) showed that

soft X-rays render water toxic to protozoa, and that this toxicity results from the formation of H_2O_2. These workers also say that, in the presence of agar, the water is not rendered toxic to protozoa. When 10 ml. of water were irradiated under conditions identical to those in experiment 2 and a medium of the same composition subsequently made from the irradiated water, this medium appeared unaffected, i.e., there was no difference in the growth of *B. subtilis* on this medium and on a control medium. Also the time of irradiation could be doubled and still no inhibition occur.

The irradiation of the small quantities of beef extract, agar, or sodium chloride used in 10 ml. of this medium, presents the problem of irradiating small volumes of the materials under the same conditions as applied to the whole medium. It is commonly assumed that X-ray effects result from the part of the radiant energy absorbed by the irradiated material and not from the part transmitted, so that the problem arises of how to adjust the dosage so as to be certain that these smaller volumes of individual constituents will absorb the same amount of energy as they did when irradiated as a part of the whole medium. This dosage is difficult to compute or to determine experimentally because any particular constituent, say the beef extract, is distributed throughout the medium and each particle of it is more or less shielded by the other constituents of the medium. It was therefore decided to irradiate the individual constituents, not only with the same dosage as was given the whole medium but also with much larger dosages in order to assure an absorption by a constituent, when irradiated individually, at least as great as when that same substance was irradiated as part of a completed medium. The following experiment shows the results.

Experiment 3. Of beef extract, 0.015 gram was placed in a microculture slide and well spread over the bottom of the depression. This was then irradiated at 35 kv., 20 m.a., 3.0 cm. from the focal spot for four hours. After irradiation, 10 ml. of medium were made from it and inoculated as usual. There was no apparent difference in the growth on this medium and the control.

When 0.05 gram of sodium chloride is similarly irradiated, the salt becomes quite brown, but a medium made from it

supports the growth of *B. subtilis* as well as a control medium. H. Zeller (1926) found that the addition of irradiated sodium chloride to a culture medium had no effect on the CO_2 production by yeast. He examined the action of many irradiated inorganic and organic substances and found some to retard fermentation, some to stimulate, and still others to have no effect.

The effect of irradiation of beef extract and sodium chloride when dissolved in water is shown by the following experiment.

Experiment 4. Of beef extract, 0.015 gram was dissolved in 10 ml. of water and irradiated at 38 kv., 10 m.a., 6.8 cm. from the focal spot for two hours. To this solution were then added the NaCl and agar and the mixture autoclaved for fifteen minutes at 15 to 20 pounds pressure and inoculated after cooling. There was no apparent difference in the growths which developed on the irradiated and the control media. Similarly, the irradiation of 10 ml. of water containing 0.05 gram NaCl caused no decrease in the amount of growth on a medium made from this salt solution.

Not until agar gels and dry agar were irradiated, was there any indication of inhibition of growth by the irradiation of individual constituents. The following experiment shows this inhibition.

Experiment 5. In a petri dish, 0.24 gram of agar and 12 ml. of water were autoclaved at 15 to 20 pounds pressure for fifteen minutes. After cooling, the gel was irradiated at 38 kv., 10 m.a., 6.8 cm. from the focal spot, for two hours. The gel was then melted in the autoclave and 10 ml. pipetted aseptically into a petri dish containing the beef extract and sodium chloride which had been previously sterilized by autoclaving. The medium was then well mixed, allowed to gel, and inoculated. No colonies appeared on this medium after incubation for forty-eight hours.

If 0.2 gram of ground dry agar is placed in a micro-culture slide and irradiated for two hours at 38 kv., 10 m.a., 3.0 cm., from the focal spot, the medium made from this agar supports a normal growth of *B. subtilis*. As pointed out above, however, the total energy absorbed may be less than when this same quantity of agar is irradiated as a 2 per cent gel as in experiment 5. Therefore, the conditions of irradiation were changed to 35 kv., 20 m.a.,

and four hours, 3.0 cm. from the focal spot. When this agar was used to make a medium it was found that it had been so altered by the irradiation that it would not gel. An additional 0.2 gram of agar (not irradiated) had to be added to the medium. This medium then gelled but it would not support the growth of *B. subtilis*. Thus it is seen *that a medium which normally will support a good growth can be so affected by the action of soft X-rays on the agar alone (or the agar-water gel) that it will no longer support the growth of B. subtilis.*

III. MECHANISM OF THE INHIBITION

There seemed to be three possible mechanisms by which soft X-rays might so alter the medium that it would no longer support the growth of *B. subtilis*. 1. Production of phosphorescence. 2. Destruction of a growth-essential. 3. Production of an antiseptic substance.

1. Phosphorescence

A phosphorescence might arise either from the container or from the medium. The glass containers in which the media were irradiated were definitely known to phosphoresce in the visible spectrum for a short time after irradiation. However, since an inhibitive medium can be transferred to a new container previous to inoculation and still be shown not to support growth, this factor can be ruled out. Repeated tests have failed to reveal any phosphorescence from the medium which would affect a photographic plate. Furthermore, since a true phosphorescence is known to diminish with time, a medium which inhibits growth by virtue of its phosphorescence should regain its power to support growth if it is allowed to stand after irradiation and before inoculation. The irradiated media may be held for twenty-four hours before inoculating and they will still not support the growth of *B. subtilis*. These experiments indicate that the inhibition does *not* result from phosphorescence.

2. Destruction of a growth essential

In the medium chosen for this work, the major growth essentials are supplied by the beef extract. Since the experiments previ-

ously discussed show that irradiation of the beef extract does not cause this inhibition, it seems unlikely that the inhibition results from the destruction of a growth essential. There remained the possibility, however, that a growth-essential in the agar was being destroyed. In order to examine this possibility the following experiment was run.

Experiment 6. Of a 2 per cent agar gel, 10 ml. were irradiated for two hours at 38 kv., 10 m.a., 6.8 cm. from the focal spot. After irradiation, the beef extract, NaCl and an additional 0.2 gram of agar were added and the medium autoclaved at 15 to 20 pounds pressure for fifteen minutes. After autoclaving, the medium was well mixed, allowed to cool and gel and then inoculated and incubated. This medium would not support the growth of *B. subtilis*. If the inhibition noticed in previous experiments had resulted from the destruction of a growth essential in the agar, the addition of more agar after irradiation in this experiment should have replaced this growth essential and the resulting medium should have supported growth. Since this was not the case, the inhibition does *not* result from the destruction of a growth essential in the agar.

3. Production of an antiseptic

Thus, the inhibition of growth does not result from phosphorescence or destruction of essential food materials. We believe that an antiseptic is produced by the irradiation of the agar and present the following experiments in support of this belief.

Experiment 7. Each of two 12 ml. quantities of 2 per cent agar gel was irradiated for two hours, at 38 kv., 10 m.a., 6.8 cm. from the focal spot. After irradiation, one of these was washed in continuously flowing distilled water (about 15 l.—not sterile) for twenty hours. At the same time another 12 ml. quantity of agar which had not been irradiated was similarly washed to serve as a control. After washing, the four agars (control, washed control, irradiated, and washed irradiated) were autoclaved at 15 to 20 pounds for fifteen minutes and 10 ml. of each added aseptically to each of four petri dishes containing 0.03 gram of beef extract and 0.05 gram of NaCl (sterile), well mixed, and allowed to gel. They were then inoculated and incubated for twenty-four hours. Only the irradiated agar which had *not* been washed showed inhibition of growth. All the others showed normal growth. Washing

the irradiated agar had removed the antiseptic and restored to the medium its ability to support growth.

Experiment 8. Each of two 12 ml. quantities of a 2 per cent agar gel was irradiated as in experiment 7 and then placed in 36 ml. of distilled water for approximately eighteen hours. The two soak waters were then combined and carefully evaporated to dryness on a steam bath. Two other 12 ml. quantities which had not been irradiated were similarly handled. To these dry residues, were added beef extract, NaCl, agar and 10 ml. of water. The mixtures were autoclaved for fifteen minutes, at 15 to 20 pounds pressure and allowed to gel. Also, the soaked gels were melted in the autoclave, and 10 ml. portions added aseptically to dishes containing beef extract and NaCl, well mixed and allowed to gel. All media were inoculated and each showed a normal growth after twenty-four hours incubation at 30°C. except the medium containing the residue from the soak waters from the irradiated gels. This latter medium showed no growth over a period of eight days.

A modification of this experiment follows. If an irradiated 2 per cent agar gel is distilled under vacuum and media made from the distillate and from the residue, the one made from the distillate supports growth, while the one made from the residue does not. A medium made from the residue of a non-irradiated gel will support growth.

Early in this paper it was emphasized that we had adopted a technique whereby the entire surface of the medium was irradiated and not just a portion of a larger continuous surface. The advisability of such a technique is now seen, since any antiseptic produced in an irradiated area of the medium will probably diffuse into the non-irradiated areas. Such a diffusion may manifest itself in two ways: first, the non-irradiated portions may become unsatisfactory for the growth of the organisms or, second, the diffusion may result in a diminished concentration of the antiseptic and thus the ability of the irradiated medium to support growth may be restored. Both of these conditions can be easily demonstrated experimentally but only the first will be discussed here.

The elemental composition of a 2 per cent agar gel is such that a 0.6 mm. thickness of the gel will absorb approximately 50 per

cent of all the energy impinging upon it when irradiated with 1.5 Å. X-rays; 1.2 mm. will absorb 75 per cent; 1.8 mm. 87.5 per cent, etc. A 10 ml. quantity of gel in a 4.3 cm. petri dish is 6.9 mm. thick. Therefore, even though the entire volume may be in the direct line of the X-ray beam, the lower half of the gel must be considered practically non-irradiated. The antiseptic will be produced primarily in the top half and will reach the lower half by diffusion. This is shown in the next experiment.

Experiment 9. A disk of medium, 4.3 cm. in diameter and approximately 6.0 mm. thick, was prepared and irradiated as in experiment 7. This irradiation is also the same as that of experiment 2 in which it was shown to be sufficient to produce total inhibition of growth. Immediately after irradiation, the disk was cut along a diameter and one-half at once sliced horizontally into 3 slices of approximately equal thicknesses—2 mm. each (top, *1a*, middle, *1b*, bottom, *1c*). The second half was held for approximately five hours to permit diffusion, and then similarly sliced (top, *1d*, middle, *1e*, bottom *1f*). All six slices were then inoculated. Figure 2 shows that during the two hour irradiation period sufficient antiseptic was produced in or diffused through the upper two-thirds of the medium to inhibit completely the growth, but that there was insufficient antiseptic to inhibit completely the growth on the bottom layer (*1c*). During the five hour diffusion period for the second half, however, the concentration of antiseptic in the bottom layer (*1f*) increased to a point where growth was completely inhibited. Two control growths are included in the figure (*C-1* and *C-2*).

Further proof that there is a change in an agar medium, as a result of irradiation, which will subsequently influence growth is obtained by the following final experiment.

Experiment 10. Three 2 ml. quantities of medium, in petri dishes were inoculated. They were then irradiated at 38 kv., 10 m.a., 6.8 cm. from the focal spot for fifteen, thirty, and forty-five minutes, respectively. Immediately after each irradiation, sub-cultures were made, using sterile cotton swabs moistened with sterile saline solution onto plates containing non-irradiated medium. After incubation for twenty-four hours, both the plate irradiated fifteen minutes and the sub-culture made from the same showed scattered growth but the sub-culture exhibited more growth than the original plate. The plate

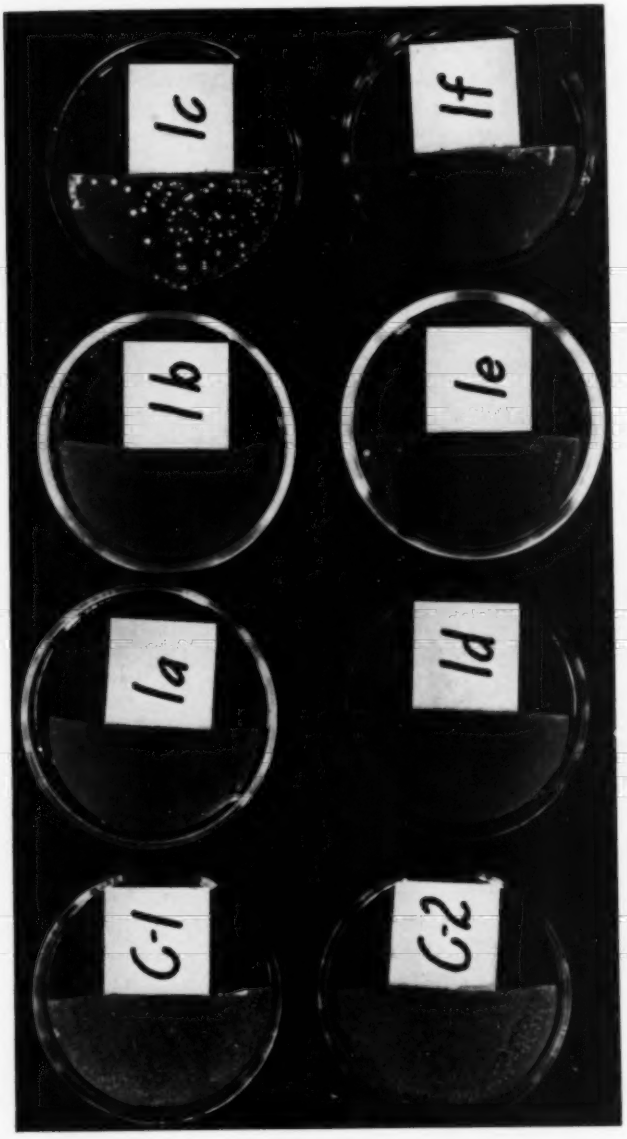

FIG. 2. SHOWING INHIBITION OF GROWTH FOLLOWING DIFFUSION (SEE EXPERIMENT 9)

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irradiated thirty minutes showed no growth but the sub-culture made from the same plate showed about 80 colonies. Neither the plate irradiated forty-five minutes nor its sub-culture showed any growth. The lack of growth on the plate irradiated thirty minutes accompanied by growth on the sub-culture made from it, indicates that the direct action of the X-rays on the organisms for thirty minutes does not kill all of them but that alteration of the medium inhibits their growth. When this same experiment was carried out using a 12 per cent gelatin gel as a medium, this phenomenon was not observed. The plate irradiated fifteen minutes and its sub-culture each showed scattered growth but the sub-culture less than the rayed plate; the plate irradiated thirty minutes showed about 50 colonies and sub-culture from it showed only 6; neither the plate irradiated forty-five minutes nor its sub-culture showed any growth. Here, where a gelatin medium was used, the sub-cultures always showed less growth than the irradiated plates from which they were made.

IV. CONCLUSIONS

We have shown that the action of soft X-rays on agar or agar-water gel alters the agar in such a manner as to render a medium subsequently made from that agar unsuitable for the growth of *B. subtilis*. This seems to result from the production of a diffusible, non-volatile material which is antiseptic to *B. subtilis*.

The demonstration of this action of soft X-rays on agar makes it essential that investigators who are irradiating organisms on the surfaces of media which contain agar differentiate between the direct action of the radiation on the organisms and the indirect action of the radiation on the agar, producing substances toxic to the organisms.

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PHYSIOLOGICAL STUDIES ON RHIZOBIUM

III. RESPIRATION AND GROWTH AS INFLUENCED BY THE REACTION OF THE MEDIUM[1]

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Several investigations have been reported concerning the effect of hydrogen ion concentration on the growth and longevity of rhizobia in the soil and in liquid cultures. Fred, Baldwin and McCoy (1932) have presented a summary of this topic. Although there are some differences of opinion, it has been found, in general, that the various species of these organisms are affected differently by acidity but have about the same tolerance to alkalinity. *Rhizobium meliloti* is the most sensitive to acidity and is favored by a neutral or slightly alkaline reaction. *Rh. lupini* and *Rh. japonicum* are the most tolerant to acidity and therefore they will grow at a lower pH than the other species of this genus.

No work has been reported concerning the specific effects of pH upon the physiological activities of these organisms other than growth or upon their ability to produce nodules on the respective host plants. This paper is a report of an investigation showing the influence of the pH of the substrate upon the growth and respiration of a strain of *Rh. meliloti* and of *Rh. japonicum*.

METHODS

The Warburg manometric technique was employed to determine the rate of oxygen consumption. This has been described

[1] Journal Paper No. J214, Iowa Agricultural Experiment Station, Ames, Iowa. Project 226. This paper covers in part the work submitted to the Graduate School of Iowa State College by the senior author as part requirement for the Degree of Doctor of Philosophy. The authors gratefully acknowledge the suggestions and criticisms offered by Dr. P. E. Brown during the course of the investigation reported here.

in detail by Walker, Anderson and Brown (1934). Growth was measured by the increase in oxygen utilization with time.

Strain 127 of *Rh. meliloti* and no. 403 of *Rh. japonicum* from the laboratory stock cultures were used. These species were chosen because *Rh. meliloti* is representative of the fast-growing species of this genus and *Rh. japonicum* is representative of the slow growers, and also because of the difference in their growth brought about by acidity. The purity and virulence of the cultures throughout these experiments were determined by microscopic examination, inoculation into skimmed milk, and the production of nodules upon the respective host plants.

Cultures for inoculation were prepared by growing the organisms in a mannitol yeast-extract liquid medium of pH 7.0, maintained at 28°C. and shaken intermittently. After thirty-six to forty-eight hours of growth the organisms were centrifuged from the medium, washed in a neutral phosphate solution and suspended in a clear solution of the unbuffered salts of the medium employed.

The medium used in the Warburg vessels was of the following composition: 0.1 gram $CaSO_4 \cdot 2H_2O$, 0.2 gram $MgSO_4 \cdot 7H_2O$, 0.1 gram NaCl, 0.025 molar potassium phosphate salts as a buffer and 1000 cc. distilled water. Ammonium chloride, 100 p.p.m. of nitrogen, or Bacto yeast extract, 1 gram per liter, was used as a source of nitrogen. Glucose, 10 grams per liter, was added as a source of carbon for *Rh. meliloti* and arabinose in the same concentration for *Rh. japonicum*.

To obtain media of different pH values, stock phosphate buffers, differing in reaction by about 0.4 pH and ranging from pH 3.8 to 10.0, were employed. They were prepared by mixing 0.2 molar solutions of H_3PO_4 and K_3PO_4 in varying proportions. The phosphate buffers, mineral salts and sugar were each sterilized separately and combined aseptically after cooling.

After inoculation of the organisms in equal numbers into equal volumes of the media of different pH values the respiration was measured in the manometers at 28°C. ±0.01°. Readings were taken at one-half hour intervals over a period of six hours for *Rh. meliloti*. Inasmuch as *Rh. japonicum* grows more slowly in

synthetic media, respiration measurements were made over a twelve-hour period.

The cultures were prepared in duplicate. One was used for the respiratory studies in the Warburg vessels, the other was used in making the pH determinations at the beginning of the experiment. At the conclusion of the experiment the pH of the cultures employed in the respiration experiments was immediately determined. The quinhydrone electrode was used for determinations below pH 8.0. Above that value, colorimetric tests were made with a Hellige Comparator. The accuracy of these determinations was checked with the glass electrode.

Changes in the pH of the media during the course of the experiments were very small except in those cultures having an initial pH above 8.0. In this pH region the buffers were less efficient and CO_2 absorption by the media was greater. In the cultures with an initial pH of 8.0 or slightly greater a decrease of about 0.1 pH unit occurred during the course of the experiments. This decrease was larger with increasing alkalinity. Cultures of pH 9.0 to 9.5 decreased as much as 0.3 pH unit. For this reason the determinations in the more alkaline media were subject to greater error than those made in the neutral or acid media. In each case the final pH values were used in the interpretation of results.

RESULTS

The results obtained in this study have been summarized and are shown graphically in the figures that follow. The points on each curve represent the average of the results obtained in five to eight separate determinations.

The first series of experiments was conducted with *Rh. meliloti*, using ammonium chloride as the source of nitrogen. The results of these experiments are shown in figure 1. The oxygen consumed between the fourth and sixth hours was taken for comparative purposes. During this period the organisms reached their maximum growth rate. The differences in the respiratory rate were not great between pH 6.2 and 7.8 and the maximum respiratory rate was at pH 7.0. No attempt was made to determine the critical reactions for growth but an examination of the rate curves

of oxygen utilization in the acid range shows that at pH 4.6 and below the rate of respiration decreased with time. The oxygen uptake was very small in the acid range and was negligible below pH 4.0.

The rate of increase of respiration by bacterial cultures has been found to correlate very closely with growth. This indicates that under optimum growth conditions the rate of respiration per viable cell approximates a constant. During the logarithmic

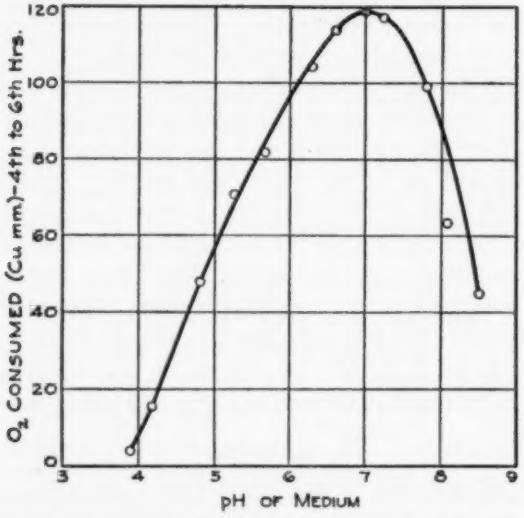

FIG. 1. OXYGEN CONSUMED BY RHIZOBIUM MELILOTI AS INFLUENCED BY THE REACTION OF THE MEDIUM

growth period, then, the logarithms of the cubic millimeters of oxygen utilized per unit period plotted against time should give a straight line of the same slope as that obtained by plotting the logarithms of numbers of organisms against time. This procedure furnishes a convenient method for the determination of K, the velocity coefficient of growth.

The data presented in figure 1 represent a combination of both respiration and growth. In studying these data about a year after their determination it was deemed advisable to substantiate

them by differentiating the effects on growth from those on respiration by the determination of K for the cultures at the various reactions. In order to determine the logarithmic growth phase in the cultures which had a reaction not optimum for growth it was necessary to increase the rate of growth to a greater extent than that observed in the first series of experiments. Hence a second series of experiments was conducted in which yeast extract, 1 gram per liter, was introduced into the medium in place of ammonium chloride as the source of nitrogen.

The logarithms of the rates of respiration during the logarithmic growth period have been plotted against time and the graphs are shown in figure 2. The influence of pH upon growth is shown by the slopes of the respective lines, and upon the rate of respiration by the relative position of the lines in the graph. These lines also indicate the critical reactions, below which a negative slope is shown. Accordingly growth is seen to cease near pH 4.6 and 9.65.

From the data of figure 2, the values of K were calculated by the formula,

$$K = 1/t \times \ln b/B$$

in which B = the cubic millimeters of oxygen utilized per one-half hour at the beginning of time t, and b = the cubic millimeters of oxygen utilized per one-half hour at the end of time t, the interval t being taken during the logarithmic period of growth.

The growth curve obtained by plotting the values of K for the various reactions is shown in curve A of figure 3. This curve gives a more complete picture of the growth of *Rh. meliloti* as a function of pH than did figure 1. The optimum pH for growth was near 7.0. From this value growth decreased with increasing acidity or alkalinity, finally ceasing between pH 4.6 and 4.9 and near 9.6. The optimum value is the same as that obtained in the first experiment. Figure 1 shows, however, that there was still some respiration even beyond those pH values at which growth stopped. The optimum was essentially the same, but the limiting acidity was found to be higher and the sensitivity towards acidity was emphasized.

A similar study was conducted with *Rh. japonicum* and the results are shown in curve B of figure 3. Inasmuch as this organism grows rather slowly in the common laboratory media the logarithmic growth phase is difficult to determine, except under the most favorable conditions. For this reason the oxygen consumed between the sixth and twelfth hours was used as a

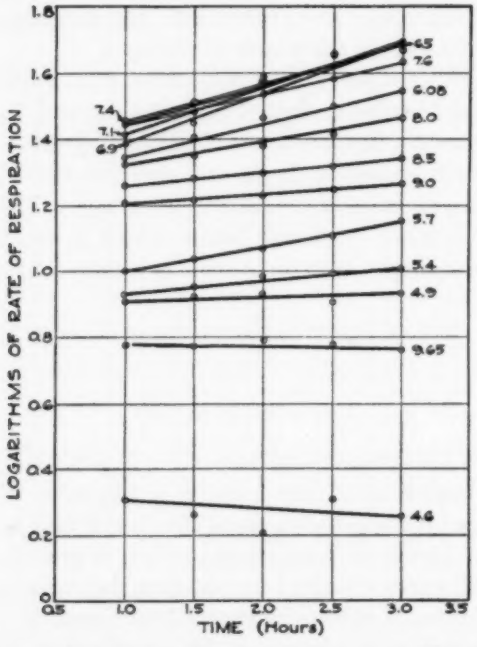

FIG. 2. THE RATE OF RESPIRATION OF RHIZOBIUM MELILOTI (CUBIC MILLIMETERS PER 0.5 HOUR) DURING THE LOGARITHMIC GROWTH PERIOD AS INFLUENCED BY THE pH OF THE MEDIUM

measure of respiration and growth. Inasmuch as the growth had reached its maximum during this period the respiration values obtained are indicative of the amount of growth that had occurred. In order to give as complete a picture as possible the data were analyzed and the points at which there was a decrease in the respiratory rate with time were determined. These points, pH

4.2 and 9.5, were located on the graph as those at which activity had ceased. The figure thus represents the effect of pH on both respiration and growth.

The optimum pH was lower than that observed for the alfalfa organism, being at about pH 6.7 to 6.9. From this optimum the activity of the organisms decreased to zero for growth at pH 4.2 on the acid side and at 9.5 in the alkaline range.

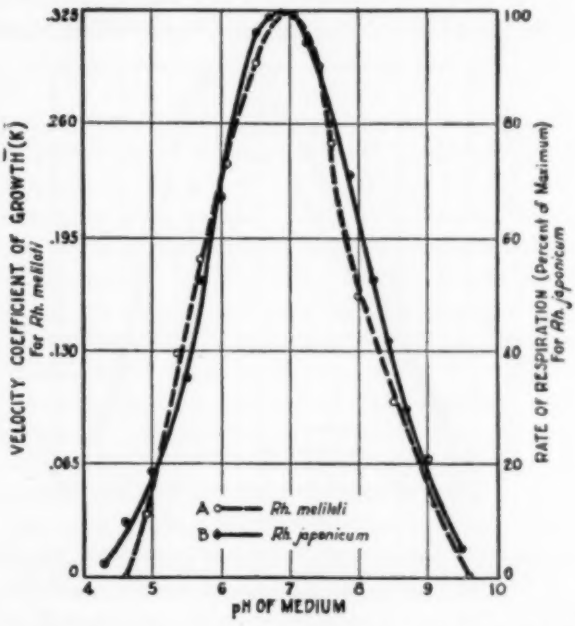

FIG. 3. GROWTH OF RHIZOBIUM MELILOTI (CURVE A), AND THE RATE OF RESPIRATION OF RHIZOBIUM JAPONICUM BETWEEN THE SIXTH AND TWELFTH HOURS (CURVE B) AS INFLUENCED BY THE pH OF THE MEDIUM

In order to determine the more specific effect of pH on respiration as distinguished from growth as was shown in figure 3, the data of the last two experiments for oxygen utilization during the first hour after inoculation have been employed. The average values have been plotted and are shown in figure 4. Inasmuch as there was an equal number of cells in all cultures in each partic-

ular test at the beginning of these experiments, and as there was an initial lag period following inoculation, growth was maintained at a minimum. Hence the oxygen consumed was almost entirely the resultant of respiration rather than growth. By this method of analysis it is possible to determine the effects of pH on respiration in the same cultures and under conditions identical with those in which the effects on growth were determined.

The curves of figure 4 show that the respiration of the two organisms responded in almost the same manner to changes in the

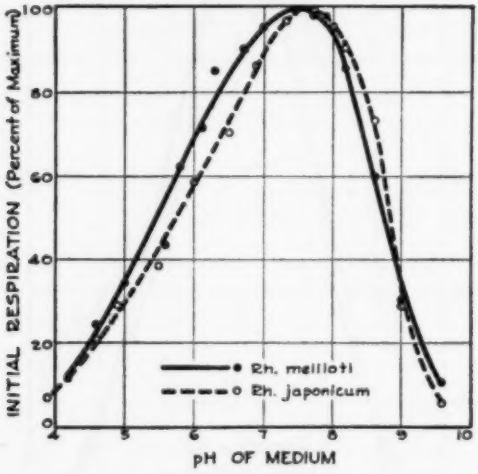

FIG. 4. THE RATE OF RESPIRATION OF RHIZOBIUM MELILOTI AND RHIZOBIUM JAPONICUM DURING THE FIRST HOUR AFTER INOCULATION AS INFLUENCED BY THE REACTION OF THE MEDIUM

reaction of the medium. In comparison to growth the optimum for respiration was in each case more alkaline, shifting from near neutrality for growth to rather a wide optimum for respiration which was between pH 7.2 and 8.0. Some respiration continued below the critical pH values although the rate decreased rather rapidly with time.

DISCUSSION

The values obtained for the optimum and critical reactions of these organisms agree substantially with those reported by other

investigators. Fred and Davenport (1918) reported the critical acidity for *Rh. meliloti* as pH 4.9 and for *Rh. japonicum* as pH 3.3. They found that all species of this genus have a similar tolerance to alkalinity. No growth was observed above pH 9.6. Wright (1925) found, on the other hand, that the critical acidity for growth of the soybean organisms varied from pH 4.2 to 4.6. No growth was reported in cultures more alkaline than pH 8.0.

From the slopes of the logarithmic growth curves of *Rh. meliloti* in figure 2 it may be noted that for the conditions of this experiment growth was very slight at pH 4.9 while at 4.6 conditions were distinctly unfavorable for growth. For the strain of *Rh. japonicum* studied growth was found to cease at pH 4.2.

An analysis of the initial respiration of the first series of experiments, in which ammonium chloride was used as a nitrogen source, gave a curve which agreed with that for *Rh. meliloti* presented in figure 4 in which yeast extract was used as the nitrogen source. Thus the suggestion that there is a difference between the optimum pH for respiration and for growth is substantiated for two species of organisms in media containing two different sugars and two different sources of nitrogen.

Enzyme actions are, in general, quite sensitive to the reaction of the substrate. Respiration is essentially an enzymic process; the rate of respiration of various organisms, however, has been found to differ rather widely as a function of pH. Burk, Horner, and Lineweaver (1934) found that the rate of respiration of Azotobacter was greatly influenced by changes in acidity. Cook and Stephensen (1928) reported, conversely, that the oxygen uptake of *Escherichia coli* is not affected to any appreciable extent between pH 4.0 and 8.0.

The rate of respiration of two species of Rhizobium was found to be quite sensitive to changes in reaction. The similarity of the two curves in figure 4 for initial respiration indicates a close relationship between the respiratory mechanism of the two species. It is further evident that the respiration is at least as tolerant to changes in reaction as is the growth of the respective organisms.

SUMMARY AND CONCLUSIONS

The respiration and growth of representative strains of *Rh. meliloti* and *Rh. japonicum* as a function of the pH of the medium were measured by means of the Warburg manometric technique.

The growth of *Rh. meliloti* in glucose yeast extract media was found to decrease from a maximum at pH 7.0 to zero between pH 4.6 and 4.9 on the acid side of neutrality and pH 9.6 in the alkaline range.

The growth of *Rh. japonicum* in arabinose yeast extract media decreased from a maximum at pH 6.7 to 6.9 to zero at pH 4.2 and 9.5.

The initial rate of respiration for the two species reached a maximum between pH 7.2 and 8.0 and decreased to a very small value at the critical pH for the respective organisms. In both cases the optimum reaction for respiration is more alkaline than that observed for growth.

Since the respiration of the two species responded almost identically to changes in the reaction of the medium it is concluded that there is probably a close relationship in their respiratory mechanism.

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STUDIES ON THE SOLUBILITY OF PNEUMOCOCCUS IN SAPONIN

III. THE SAPONIN-LYSIS REACTION AS A MEANS OF DIFFERENTIATING PNEUMOCOCCUS AND STREPTOCOCCUS

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In previous communications (Klein and Stone (1931), Klein (1933a, 1933b)) we reported that under certain specified conditions the pneumococcus is dissolved by saponin. In the present paper we wish to detail certain observations which indicate the applicability of this reaction to the differentiation of pneumococci and streptococci. Fourteen strains of pneumococcus (including rough and smooth strains of pneumococcus types I, II, III and group IV) and eight strains of *Streptococcus hemolyticus* and *Streptococcus viridans* were tested for solubility in saponin. All the pneumococcus strains were found to be saponin-soluble, while the streptococcus cultures were all saponin-insoluble.

The tests for saponin bacteriolysis were performed as previously described (Klein and Stone, 1931). The saponin employed in these experiments was *saponin Merck pure*. We may restate here that in order to demonstrate the dissolution of pneumococci by saponin it is essential that there be added to the culture media either a sterol (cholesterol, ergosterol) or animal fluid such as blood or ascitic fluid. In the absence of these "sensitizing substances" no lysis is obtained when the standard beef-infusion broth is used as culture medium.[1] Evidence has been presented

[1] Downie, Stent and White (1931 Brit. Jour. Exper. Path., **12**, 1) reported that a strain of pneumococcus was lysed by "sapotoxin" when tested in hormone broth culture. It is not possible to evaluate this observation in the absence of any information as to the nature of the sapotoxin, the cholesterol content of hormone broth, and the amount of blood carried over into the culture medium.

to show that it is the cholesterol contained in these animal fluids which affects the sensitization of the pneumococci to saponin; furthermore, that the bacteriolysis depends upon a union of saponin with the cholesterol adsorbed by the bacteria during the sensitization.

In our first communication, as a result of our earliest observations, we divided our 13 test strains of pneumococci into two groups: "reactive" (9 strains) and "non-reactive" (4 strains). The term "reactive" denoted those strains which were found to be saponin-soluble when grown in broth containing animal fluid. No correlation was apparent between "reactivity" and pneumococcus type or colony morphology. Two of the 4 non-reactive strains were maintained in our culture collection for two years. It occurred to us that we had never tested the non-reactive strains with cholesterol instead of animal fluid as the sensitizing agent in the culture medium; all experiments on cholesterol sensitization had been performed with the 9 reactive strains. The 2 so-called non-reactive strains were then tested for saponin-solubility in 0.01 per cent cholesterol broth, 2 per cent serum broth, and plain broth culture. The cholesterol broth cultures were completely dissolved by saponin 1:20 in forty minutes at room temperature and by saponin 1:1,000 after refrigeration overnight. The serum broth cultures also showed lysis which, however, was somewhat slower than in the case of cholesterol broth cultures. The plain broth cultures showed no lysis after two hours at room temperature and refrigeration overnight. We are unable to explain the apparently changed reactivity of these strains to animal fluid. It is possible that the negative results originally obtained with these strains were in some way associated with the fact that ascitic fluid was used as the sensitizing substance in these earlier tests. The important consideration was that all pneumococcus strains tested in cholesterol broth culture proved to be saponin-soluble. Hence the possible value of the reaction in differential diagnosis.

Eight strains of streptococci were tested for saponin-solubility in plain, cholesterol, and serum broth culture. None of the cultures gave any macroscopic or microscopic evidence of lysis

after standing for two hours at room temperature and overnight in the refrigerator. The concentration of reagents used in these tests was as follows: cholesterol 1:5,000, 1:50,000; blood serum 1:10, 1:20; saponin (final dilution) 1:20, 1:100; bacteria—twenty-four-hour culture, diluted 1:1 and/or 9:1 with solution of saponin in saline.

TABLE 1

*Comparative lysis of rough and smooth variants of pneumococcus type I**

TIME OF INCUBATION	0.5 CC. OF CULTURE + 0.5 CC. FOLLOWING REAGENT				
	Saline	Saponin 1:500	Saponin 1:10	Bile 1:10	Bile undiluted
Rough variant					
½ hour at room temperature	No lysis†	Moderate to strong	Moderate to strong	No lysis	Weak
1½ hour at room temperature	No lysis	Strong	Strong	Moderate to weak	Strong
22 hours in refrigerator	No lysis	Almost complete	Almost complete	Almost complete	Almost complete
Smooth variant					
½ hour at room temperature	No lysis	Complete	Complete	Strong	Complete
1½ hour at room temperature	No lysis	Complete	Complete	Strong	Complete
22 hours in refrigerator	No lysis	Complete	Complete	Complete	Complete

* The bacteria were tested in eighteen-hour culture in 0.01 per cent cholesterol broth. The saponin was used in saline solution. The bile (Bacto-Ozgall Difco, Digestive Ferments Co.) was dissolved in distilled water to give a concentration corresponding to whole bile; subsequent dilutions were made in saline.

† The degree of lysis was recorded as: no lysis, trace, weak, moderate, strong, almost complete, complete.

All tests were controlled by parallel bile solubility tests. Certain rough pneumococcus strains are dissolved by bile only slowly and in high concentrations of the reagent (Reimann, 1927). In fact, some authors (Kimura, Sukneff and Meyer, 1928; Neufeld, 1900; Neufeld and Händel, 1908) report that certain avirulent strains are insoluble in bile. It was therefore of interest to study the saponin-solubility of rough pneumococci. Rough and smooth strains of pneumococcus[2] types I, II, and III were tested in plain,

[2] We are indebted to Dr. M. H. Dawson for these strains. The strains were originally obtained from the collection at the Rockefeller Institute and are labelled as follows: I_S = 1/230; I_R = I/192/R; II_S = D39; II_R = D39R; III_S = A66; III_R = M3R.

cholesterol, and serum broth culture. No lysis was obtained in saponin with any of the plain broth cultures. All the serum broth and cholesterol broth cultures were found to be soluble in saponin. Only in the case of type I was a relatively increased resistance to lysis manifested by the rough variant. The results obtained with the cholesterol broth cultures of type I pneumococci are recorded in table 1. The smooth variant was completely dissolved by saponin within thirty minutes, whereas the rough variant was not dissolved until the next day. Even then the saponin-treated bacterial suspension failed to become perfectly clear. Significantly, a similar result was obtained with bile as the lytic agent, indicating the existence of a factor common to both bacteriolytic reactions. Another observation worth mentioning here, is that the rough variant is much more resistant to autolysis than is the smooth variant.[3] It is also apparent from the table that the relatively resistant pneumococcus type I_R is much more rapidly dissolved by saponin 1:1,000 than by bile 1:20. The serum broth cultures gave analogous results. In the case of pneumococcus type II no significant difference was detected between the rough and smooth variants as regards rate of saponin-lysis, bile-lysis or autolysis. In the case of type III, the rough variant showed more rapid lysis in saponin than did the smooth variant; this was paralleled by the more rapid autolysis manifested by the rough variant.

It is often expedient in diagnostic procedure to test the bile-solubility of cultures isolated on blood agar. It is therefore of interest to note that saline suspensions of pneumococci harvested from blood agar culture were found to be rapidly dissolved by saponin.

Several tests were performed with other species of bacteria. Ascitic fluid, defibrinated blood, blood serum or cholesterol was added to the culture media to obtain "sensitization." In no case was it possible to demonstrate saponin-solubility. The species studied included: *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Hemophilus influenzae*, *Hemophilus pertussis*, *Eberthella typhosa*,

[3] Studies on the relationship between saponin-lysis, bile-lysis and autolysis will be subsequently reported.

Aerobacter aerogenes, Klebsiella pneumoniae, Vibrio cholerae (three strains).

DISCUSSION AND CONCLUSIONS

Saponin is found to be a useful reagent in differentiating pneumococci and streptococci. No attempt is made in this place to compare its merits as a differential test with that of bile-lysis which has successfully endured the severe test of time. Certainly the number of strains tested with saponin is as yet all too few. However, in our experience, the saponin test has been found just as expedient and reliable as the bile test and has even proved its superiority in certain instances, e.g., in the case described above, of pneumococcus type I_R. Moreover, the separation of these two bacterial species is of such great practical and theoretical significance that any additional differential test may prove of importance. Certain characteristics of the saponin-lysis reaction deserve emphasis.

1. The simplicity of the "sensitization" technique.

2. The rapidity of the lysis reaction. The time required for lysis varies with the concentrations of the sensitizing substance and of the saponin. With saponin in 1:20 concentration, and cholesterol in 1:5,000 to 1:500,000 concentration, lysis often appears within a few minutes and is usually complete within thirty minutes at room temperature. The bacterial suspension becomes completely clarified and transparent. No intact bacteria are to be found on microscopic examination in the case of complete lysis.

3. The high degree of bacteriolytic power possessed by saponin. Lysis is somewhat slower with the saponin in 1:1,000 dilution; however even a 1:20,000 dilution shows strong bacteriolytic activity for "sensitized" pneumococci.

4. The ready solubility of saponin in water, and the stability of the sterile aqueous solution. The saponin solution may be sterilized by autoclaving without apparent diminution of its bacteriolytic activity, whereupon it will keep for a long time.

5. The availability and inexpensiveness of the reagents.

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TUBERCULOUS BACILLEMIA AND DEMENTIA PRAECOX[1]

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Loewenstein's method for culturing *Mycobacterium tuberculosis* directly from human blood has been the subject of intensive investigation and review throughout the bacteriologic world. Not only has Professor Loewenstein offered to examine countless specimens but he has generously given his personal attention to anyone interested in studying his methods and results in his laboratory in Vienna. We are privileged to number ourselves among those to whom he has extended unusual courtesies and wish to take this opportunity of making grateful acknowledgment.

In a previous communication (Kopeloff and Loewenstein, 1934) we reported the results of Loewenstein's examination of triplicate specimens of blood from psychotic patients and mentally normal subjects without his knowledge as to their origin. He found *M. tuberculosis* in 15 out of 34 dementia praecox and a total of 20 out of 42 psychotic patients, and none in 12 control physicians and nurses. However, in no single patient did all three blood specimens yield a positive culture. Whenever blood was taken from the above subjects, 42 cc. were withdrawn, i.e., enough to furnish *6* specimens of 7 cc. Three were sent to Loewenstein and the other 3 were cultured in our own laboratory according to Loewenstein's directions which were received in personal communications. In spite of our scrupulous adherence to the technic as outlined we failed to culture *M. tuberculosis* from any specimen.

The first series of bloods were taken between November 14, 1932, and January 13, 1933. A second series of bloods taken in the same manner between September 11 and 14, 1933, *from a selected number of the same psychotic patients* yielded in Loewenstein's hands the results shown in table 1.

[1] Presented at the Society of American Bacteriologists, Chicago, Illinois, December 27, 1934.

In no instance was a positive finding identical in both series. In two cases, one of dementia praecox, the other of involutional melancholia, macroscopic positives in the second series corresponded with "microscopic positives only" in the first series. In no single case, in either the first or second series, did all three blood specimens from the same patient yield positive results. In most instances only one positive finding was reported from triplicate samples. Thus, it may be said that there is little, if any, consistency in the findings on repeated examinations of the same patients. Again triplicate specimens obtained simultaneously and examined in our own laboratory failed to yield a single positive culture of *M. tuberculosis*.

TABLE 1

Loewenstein's results of blood culture in second series as compared with first series of same patients

DIAGNOSIS	TOTAL NUMBER OF CASES EXAMINED	SECOND SERIES	FIRST SERIES	
		Positive macroscopic	Positive macroscopic	Positive microscopic only
Dementia praecox	10	4	1	2
Psychoneurosis	2	0	0	1
Manic-depressive	1	0	1	
Involutional melancholia	1	1		1
Total	14	5		
Controls	3	0		

Following our visit to Vienna a third series of blood specimens, taken as before from male psychotic patients and controls, were obtained from November 10 to December 7, 1933. The results as reported by Loewenstein are shown in table 2. It will be noted that in this series the proportion of positive findings in dementia praecox slightly exceeds those in the first and second series. However, the proportion of macroscopic positives in the control group was even higher, indicating that the positive findings had no special significance so far as dementia praecox was concerned.

It is of interest to observe that 4 patients were in 3 series and 1 had blood taken 4 times in triplicate. More specifically only 1

positive occurred in 3 of the patients from whom 9 samples of blood were taken. One positive was reported in the 12 samples taken from the other patient. All were diagnosed as dementia praecox. While it is true that positive findings are of much greater importance than negatives in such an investigation one cannot fail to be impressed by the lack of consistency. Again, our own laboratory could not culture *M. tuberculosis* from any specimen.

TABLE 2

Loewenstein's results of blood culture in the third series

DIAGNOSIS	TOTAL NUMBER OF CASES EXAMINED	POSITIVE MACROSCOPIC
Dementia praecox	14	7
Psychoneurosis	1	0
Psychosis with tuberculosis	1	0
Mental deficiency	1	1
Total	17	8
Controls (physicians and nurses)	9	5

TABLE 3

Loewenstein's results of blood culture in the fourth series

DIAGNOSIS	TOTAL NUMBER OF CASES EXAMINED	POSITIVE MICROSCOPIC ONLY	POSITIVE MACROSCOPIC
Dementia praecox	38	1	0
Other psychoses	4	0	0
Controls	30	1	1

A fourth series of blood specimens were taken in the usual manner from January 5, 1933, to April 25, 1934 and the results are shown in table 3.

From table 3 it will be seen that positive findings of *M. tuberculosis* are conspicuous by their absence. All Loewenstein's results from psychotic patients were negative, despite the fact that of these patients 3 had previously yielded macroscopic positives. One of these was the only patient in that series in whom a positive

finding had occurred in all 3 of the triplicate blood specimens. One of the most significant features of the fourth series is that the number of controls nearly equals that of the psychotic patients, 38 of whom were diagnosed as dementia praecox. The 4 other psychotic patients were diagnosed as follows: epilepsy, manic-depressive, chronic alcoholism and one undiagnosed psychosis.

It should also be mentioned that in this series 8 psychotic female patients and 3 female controls were included. Again in our own laboratory we failed to culture *M. tuberculosis* in any blood specimen.

Finally on May 25, 1934, we sent Loewenstein a fifth series of single specimens of bloods from 25 patients in the Montefiore Hospital, obtained for us by Dr. Harry Richman, whose kind coöperation is gratefully acknowledged. All these patients were active febrile cases of pulmonary tuberculosis. Together with these we sent 25 control blood specimens taken from 5 healthy dogs. To date, April 15, 1935, no report has been forthcoming upon repeated inquiry. Since more than 10 months have elapsed we assume that all these findings were negative. Previously we had cultured 28 specimens of blood obtained by Dr. Harry Richman from 15 cases of active tuberculosis in the Montefiore Hospital. All of these proved negative for *M. tuberculosis*.

Since Loewenstein has claimed a high percentage of positive blood cultures of *M. tuberculosis* from patients with eye infections we obtained through the courtesy of Dr. Richard Thompson of the Department of Ophthalmology Research of the College of Physicians and Surgeons, New York, 50 specimens from 24 patients. These were sent to Loewenstein from November 29, 1933 to June 7, 1934. Eight patients were diagnosed as uveitis, 5 iritis, 4 iridocyclitis, 2 chronic choroiditis, 2 tuberculous retinitis, etc. All blood cultures were reported negative by Loewenstein. In our own laboratory we cultured 31 specimens of blood from 13 of these patients and again our results were entirely negative.

A summary of Loewenstein's findings on all bloods sent to him is presented in table 4. The data presented in this table offer grave difficulties in interpretation. In the first place, we must admit at the outset that the total number of cases in each of the

groups is relatively small. Secondly, the different groups are of unequal size. Finally, we are at a loss in evaluating the significance of microscopic findings alone. A consensus of the best bacteriologic opinion would doubtless fail to recognize the validity of such findings as being sufficient proof of the presence of tubercle bacilli in the blood stream. In our opinion they might well be acid-fast saprophytes of adventitious origin.

Even positive macroscopic findings are not generally acceptable as final criteria. We therefore requested Loewenstein to send us *all* positive cultures obtained from bloods shipped by us. We received 26, of which 24 proved to be *M. tuberculosis* by guinea pig inoculation. One of the 2 cultures which failed to infect guinea pigs was received in a desiccated state.

TABLE 4

Summary of Loewenstein's results of blood culture

DIAGNOSIS	TOTAL NUMBER OF CASES EXAMINED	POSITIVE MICROSCOPIC ONLY	POSITIVE MACROSCOPIC
Dementia praecox	96	6	21
Other psychoses and neuroses	19	2	5
Active tuberculosis	25	0	0
Eye infections	24	0	0
Controls	60	0	5

In table 4 it was recorded that there were 21 macroscopic findings in 96 dementia praecox patients, or roughly one-fifth. In the "other psychoses and neuroses" there were 5 macroscopic positives in 19 patients or about one-fourth. And there were 5 macroscopic positives in 54 controls or roughly one-eleventh. If microscopic positives are accepted as diagnostic proof then *M. tuberculosis* was found in slightly more than one-fourth of the other psychotics and neurotics; and in one-ninth of the controls.[2]

Because of the failure to obtain macroscopic positives in our own laboratory we resorted to measures other than the routine

[2] Loewenstein also reported 6 positive findings by direct smears of blood. Five of these were from patients who yielded positive macroscopic cultures and one from a control, otherwise negative.

Loewenstein method for culturing blood. Slant washings of at least one tube from each blood sample in the first series of patients were injected into normal guinea pigs. No tuberculous lesions were found. Again, 150 tubes from this series were transferred 4 to 6 months later to fresh Loewenstein medium. Twenty-one tubes were transferred also to Petroff's gentian-violet egg medium. After 5 months all tubes were retransferred to Loewenstein medium. Similarly 51 slants belonging to the second series (see table 1) were transferred 2 months after the original seeding to Loewenstein medium. All results remained consistently negative.

A word might be said at this point concerning the importance of microscopic findings alone. We made an exhaustive microscopic study of the slants from the 165 blood specimens in the first series of 54 subjects. This examination included all tubes under cultivation and was made after 4, 6, 8, 12, and 16 weeks respectively until a positive finding was obtained. In one or more slants from each specimen we found at least one acid-fast rod regardless of whether the sample came from a patient or normal control. Seldom were more than three such structures found in an entire smear. Since painstaking examination yielded a positive result microscopically in every case, we discontinued this method of approach. However, it was of interest to reëxamine microscopically a number of tubes that still appeared negative after one year's incubation. Of 8 subjects whose original slants were examined, 2 were found to contain many typical acid-fast bacilli and in 12, whose transfers made after 5 months were examined several months later, 4 were microscopically positive and 8 negative. Transfers from all positives failed to yield macroscopic growth. Because of the occurrence of these microscopic positives the contents of all tubes from the first series of patients including all transfers, were retransferred by slant washings to fresh medium. In no case did a macroscopic growth of *M. tuberculosis* develop even after prolonged incubation.

In order to control the cultural procedure the contents of 111 tubes in which physiologic saline was substituted for human blood were routinely cultivated by the Loewenstein method. After more than one year's incubation all tubes remained negative.

Loewenstein was kind enough to send us 18 sealed tubes of his medium. Half of these were incubated for sterility and the remainder inoculated with the blood sediment from 3 patients with active pulmonary tuberculosis. After 3 months' incubation no growth of *M. tuberculosis* was observed in the inoculated tubes and after 4 months' incubation the uninoculated tubes remained sterile.

Since we had failed by the Loewenstein method to obtain a single positive macroscopic growth of *M. tuberculosis* we cultivated large amounts of blood (165 to 550 cc.). Seven such specimens were obtained from patients with dementia praecox (3 of whom had been recently reported macroscopically positive by Loewenstein). The major part of each blood specimen was divided into 2 equal portions. The sediment from one portion was inoculated on Loewenstein medium and the other sediment was injected into 2 guinea pigs. Simultaneously attempts were made to cultivate the remainder of the blood by the Busson (1933) and Popper (1933) methods. Twenty-four specimens of 21 cc. of blood from 15 patients with dementia praecox and 9 controls were likewise cultured simultaneously by the Loewenstein, Busson and Popper technics. We sent Loewenstein the usual triplicate samples from all of the above subjects. Our results were entirely negative. His report was also negative except for one microscopic positive from a patient with dementia praecox.

There is another aspect of the general problem which is stressed by Loewenstein, namely, the cultivation of tubercle bacilli from spinal fluid. While we would have preferred to examine blood and spinal fluid simultaneously from each subject, this was not practicable. Logically the Loewenstein method of spinal fluid culture should prove of value in the diagnosis of tuberculous meningitis. Through the kind coöperation of the members of the staffs of Dr. W. W. Palmer of the Presbyterian Hospital, Dr. R. McIntosh of the Babies Hospital and Dr. F. Tilney of the Neurological Institute, all in New York, we cultured 8 spinal fluids from patients diagnosed as tuberculous meningitis. Six of the 8 cultures yielded a typical macroscopic growth of *M. tuberculosis*. Direct guinea pig inoculation of spinal fluid from the 2 cases

which failed to give macroscopic growth of tubercle bacilli also proved negative. Spinal fluid obtained from 5 patients with no evident clinical signs of tuberculosis proved negative on culture. Likewise spinal fluids from one patient with pulmonary tuberculosis, one patient diagnosed as pulmonary tuberculosis with brain involvement, and one of intestinal tuberculosis proved negative on culture as did spinal fluids from one patient with dementia praecox and 2 patients without psychosis (epilepsy, psychopathic personality).

These positive cultural findings in patients suspected of tuberculous meningitis were obtained after 16, 23, 27, 52 and 125 days respectively. Incidentally, the ease with which such macroscopic cultures of *M. tuberculosis* were isolated attests to the high quality of the Loewenstein medium.

DISCUSSION

Loewenstein's methods and results have been subjected to considerable adverse criticism. We shall not attempt to review the literature or analyse his results as Wilson (1933) and others have already done so. Prior to this investigation Loewenstein (1933) claimed 80 per cent positive cultures from the blood of febrile cases of pulmonary tuberculosis and 40 per cent positive cultures from the blood of 359 cases of dementia praecox. In 222 ophthalmologic cases there were 16 per cent positive findings. Loewenstein has repeatedly stated that he has never obtained a positive blood culture of *M. tuberculosis* from controls. Our chief concern here is with the results obtained by him on blood samples sent by us *where the diagnosis was withheld until his final report was made.* In general, triplicate samples of blood were sent from each patient or control.

Contrary to Loewenstein's previously published results he has now reported 5 positives in 54 controls and *no* positives in 25 cases of active pulmonary tuberculosis and in 24 cases of eye infection. The discrepancy between Loewenstein's present and former results therefore raises a serious doubt as to the validity of his claims. Of the 5 healthy young male controls which yielded a macroscopic growth of *M. tuberculosis* 3 were doctors, one a

medical student and one a nurse. One medical student yielded a microscopic positive. None of these men had ever shown any symptoms of tuberculous or rheumatic infection and their family histories were likewise negative except in one instance of rheumatic fever in a father and another instance of tuberculosis in grandparents.

In dementia praecox Loewenstein now reports 28 per cent of 96 cases positive as compared with his previous claim of 40 per cent. The difference may be accounted for by the smaller number of cases in the present series. However, it is striking that the positive findings fall into definite groups with regard to time. Thus, in the first series of 34 dementia praecox patients, 15 were positive; in the second series 4 were positive out of 10; in the third series 7 were positive out of 14 and in the fourth series none were positive in 38. All samples were sent from September to April of each year.

Loewenstein reported here 7 positives from 19 cases of miscellaneous psychoses and neuroses, or about the same proportion as in dementia praecox. Surely on the basis of these findings there is no evidence for singling out dementia praecox as a mental disease in which positive blood cultures have significance as Loewenstein has claimed.

Finally, it will be remembered that the triplicate specimens of blood sent Loewenstein represented a single withdrawal of blood divided into 3 parts. Forty cases were reported by him as positive macroscopically or microscopically. In 32 of these cases only 1 of the triplicates was reported as positive; in 7 cases 2 of the triplicates were positive and in only 1 instance were all of the triplicate specimens positive. The obvious failure to obtain consistent results on 3 equal portions of the same blood sample constitutes in itself a serious criticism of his method.

Our own cultural studies with blood from the same subjects have yielded consistently negative results. It might be argued that we failed to master some of the more subtle intricacies of the procedure. In order to compensate for any such deficiencies we visited Loewenstein's laboratory in Vienna during the summer of 1933. Prior to that visit, at Loewenstein's written suggestion, we

substituted glucose for potato sugar which we then employed subsequently. Also we had adjusted the pH of our medium to 7.4 since this point had never before been mentioned, but afterwards this practice was omitted. Before our visit we centrifuged at 2500 r.p.m. for 25 minutes, later at 1500 r.p.m. for 30 minutes. In short, we followed carefully all of Loewenstein's directions. After our visit we purchased all chemicals and dyes from the same sources as did Loewenstein. So far as we could determine our only discrepancies were our failure to use Viennese eggs and Viennese water.

SUMMARY

1. In most instances *triplicate* samples of blood from patients with mental and physical disease as well as from control subjects were sent to Loewenstein as individual specimens of unknown origin. Among 54 controls 5 positive macroscopic cultures of *M. tuberculosis* were reported, coming from healthy young males in whom there had been no demonstrable tuberculous infection. *No* macroscopic cultures of *M. tuberculosis* occurred in 25 cases of active pulmonary tuberculosis or 24 cases of eye infection (2 of which were diagnosed tuberculous retinitis). Twenty-seven positives were reported in 96 cases of dementia praecox and 7 positives in 19 miscellaneous psychoses and neuroses.

2. The above data contradict Loewenstein's previous claims of: (*a*) a high incidence of positive blood cultures of *M. tuberculosis* in febrile pulmonary tuberculosis and (*b*) in dementia praecox; and (*c*) a complete absence of positive findings in controls.

3. In our own laboratory by rigidly following Loewenstein's technic on 375 blood samples from the same patients we were unable in any instance to culture *M. tuberculosis*.

4. Of 8 spinal fluid specimens from suspected cases of tuberculous meningitis 6 yielded positive cultures of *M. tuberculosis* on Loewenstein medium in 16, 23, 27, 45, 52 and 125 days respectively.

5. Loewenstein's results reported here and our failure to obtain any positive blood cultures indicates that there is no justification for our further study of patients with dementia praecox by his methods.

It is indeed difficult to express adequately our appreciation to the many who have aided us in this investigation. We are particularly indebted to Dr. C. O. Cheney, Director, who has followed our work closely and given us the benefit of his counsel at every turn. Dr. J. R. Blalock, Dr. S. E. Katz and our nursing staff have rendered invaluable service with regard to the mentally diseased patients. We are also indebted to the volunteers who donated their blood, many of them third year medical students in the College of Physicians and Surgeons, New York. To Dr. M. M. Steinbach we extend our thanks for his generous assistance.

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STUDIES OF FRESHWATER BACTERIA

II. STALKED BACTERIA, A NEW ORDER OF SCHIZOMYCETES[1]

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"Pour se faire une idée suffisante sur les relations taxonomiques des bactéries, groupe devenues si important sous tant des rapports, il convient de ne pas se borner aux formes les plus habituelles et à cause de cela les mieux connues; il faut diriger les recherches sur des bacteries particulières, capables de donner quelques aperçus nouveaux sur la question de la morphologie du group en général."—ÉLIE METCHNIKOFF.

In the first paper of this series (Henrici, 1932) there were illustrated bacteria growing upon submerged slides which appeared to be attached to the slide by stalks. Since the publication of that paper a number of further studies have been made, in the course of which further types of stalked bacteria have been found. It has been determined beyond question that the fine filaments to which the bacteria are attached are actually stalks, because holdfasts have been found at their bases. A search of the literature has revealed descriptions of other types of stalked bacteria. While our study of these organisms is necessarily very incomplete, we feel that we have sufficient data on hand to warrant a preliminary discussion of these peculiar bacteria and their taxonomy.

To one unacquainted with these microörganisms, they might at first glance be considered as types of Myxobacteriales, which are also stalked bacteria in the sense that they form fruiting bodies upon stalks. The mechanism by which the stalks are formed in the two groups is, however, quite different. In the myxobacteria,

[1] Aided by grants from the Graduate School of the University of Minnesota, and from the National Research Council.

the stalks are at first composed of bacterial cells, which arise by peculiar creeping movements of one cell upon another; the stalks are of macroscopic dimensions, and bear a very large number of cells at their tip. With the organisms here considered, each bacterial cell is borne upon an individual stalk, which is composed of gum or iron, and which is produced by a continuous secretion from one particular portion of the cell. They are exactly analogous to the stalked diatoms and protozoa. After cell division, the stalks may branch, so that in some cases a rather elaborate colony is eventually formed.

Such bacteria have been almost completely unknown to science because the standard methods of bacteriology will not reveal their presence. They cannot readily be fitted into any of the existing orders of bacteria. They are distinctive in their morphology, and a number of types have been found, sufficient to warrant the creation of a new order to include them. We propose the creation of such an order, to be called Caulobacteriales, from the Greek καυλός, a stalk.

STALKED BACTERIA PREVIOUSLY DESCRIBED

The following bacteria have previously been described as forming stalks: *Nevskia ramosa* Famintzin, *Bacterium pediculatum* Koch and Hosaeus, *Gallionella ferruginea* Ehrenberg, *Gallionella minor* Cholodny, and *Hyphomicrobium vulgare* Stutzer and Hartleb. A *Pasteuria ramosa* described by Metchnikoff, while not so distinctly stalked, shows strong morphologic similarities to certain of our stalked bacteria, and may well be included in the group. An unnamed organism described by Mabel Jones as possessing a single polar flagellum visible in slides stained by ordinary methods, was probably a stalked bacterium. The same, or a very similar organism, was reported by Omeliansky, and named *Bacillus flagellatus*. We believe that the *Vibriothrix tonsillaris* recently described by Tunnicliff and Jackson is also a stalked bacterium.

Nevskia ramosa

This organism was observed in 1891 by Famintzin in an aquarium in the botanical gardens at St. Petersburg. It formed

a scum on the surface of the water, composed of globular, bush-like or plate-like colonies. The colonies were composed of gummy material arranged in dichotomously branched stalks arising from a common base, with the bacterial cells contained in the gum, a single cell at the tip of each stalk. The rod-shaped cells were set with their long axis at right angles to the axis of the broad lobe-like stalks.

The cells averaged 12μ in length, the length being 2 to 6 times the thickness. A distinct membrane could be demonstrated by staining with weak methyl violet, and in some instances concentric layers of capsular material were found surrounding the cells. Within the cells were highly refractile globular bodies varying in number and size. These were completely soluble in 70 per cent alcohol, and were thought to be composed of ethereal oils. The stalks dissolved rapidly in 1 per cent sodium hydroxide, setting free the cells which could then be readily stained and observed.

The cells multiplied by transverse binary fission, each daughter cell continuing to secrete its stalk, which thus produced the forked branching. At times the cells were seen to be set free from the stalks and to float away in the water. In this way the species is probably spread, such freed cells starting new colonies. All attempts at cultivation were unsuccessful.

Famintzin compared his organism to the palmellaceous alga, Urococcus, which similarly forms colonies of branched lobose stalks with the cells enclosed in gum at the tips, while Migula notes a resemblance to *Chaetophora endiviaefolia*. One might at first glance believe that Famintzin was dealing with an alga of some sort. The size recorded is rather large for bacteria, but well within the limits of known bacteria. In cells as large as this, green pigment should be observed if present. The complete lack of internal structure other than the fat globules is distinctly a bacterial character. The marked resemblance to *Bacterium pediculatum* Koch and Hosaeus, which is distinctly a bacterium, strengthens the opinion that the organism described by Famintzin is truly a bacterial species.

We have recently encountered an organism practically identical

with *Nevskia ramosa* in morphology, save for its smaller size; the cells average about 1μ in thickness and 3 to 4μ in length. It appeared as a slimy scum upon the surface of a jar of water from the lily-pond in the University greenhouse, to which we had added a little sodium sulphide to encourage a growth of Beggiatoa. The new organism appeared intermingled with numerous Beggiatoa threads, but upon transfer to new sterilized flasks of the same water with sodium sulphide added, it has been possible to obtain a continued growth of the stalked bacterium in serial cultures with a relative decrease of the other forms. The cells contain each a number (3 to 4 on the average) of highly refractile vacuoles which resemble the fat vacuoles described by Famintzin, but these do not stain with scarlet red. The fact that the organisms are distinctly favored by the addition of sulphide to the medium makes it strongly presumptive that they are sulphur bacteria, and that the vacuoles contain sulphur in the same form in which it appears in Beggiatoa. This organism is still under investigation.

Beauverie has published an illustration of a zoögloea of *Azotobacter chroococcum* in which the cells are radially arranged about a mass of gum, each cell or pair of cells occurring at the tip of a broad lobe of gum resembling very closely the arrangement of the cells in *Nevskia ramosa*. Such a structure must, however, be exceptionally rare in Azotobacter, or it would have been recorded by others. We have examined many preparations of this organism, especially in slides negatively stained with Congo red, which shows the distribution of the slime, and have never observed such a stalked structure.

Bacterium pediculatum

This organism was found growing in the syrup of a sugar refinery as zoögloeae, macroscopically resembling those of *Leuconostoc mesenteroides*. Microscopic examination of these masses showed them to be composed of twisted, short, thick, sausage-like filaments, often branched. But heated, stained smears showed only short, fine rod forms. Examination of wet unheated material in a dilute methylene-blue solution explained the dis-

crepancy. The rod forms were found growing at the tips of the thick thread-like structures. At the tip of each was found a single cell, with its long axis at right angles to the axis of the stalk, the stalk of "kolossalen" dimensions as compared with those of the bacterial cell. The cells multiplied by transverse fission, and each cell continuing to secrete gum on one side, the stalks became branched. The gum of which the stalks are composed is easily soluble on heating.

No dimensions are given, but from the drawing and description one gains the impression that the cells were of ordinary bacterial dimensions, say about the size of a colon bacillus. Koch and Hosaeus state that their organism shows some resemblance to certain stages of the *Bacterium vermiforme* of Marshall Ward. They were unable to obtain cultures.

Gallionella ferruginea

This is a very remarkable bacterium which has undergone many misadventures with regard to nomenclature and classification. It was first described by Ehrenberg, who placed it in the algal genus Gaillonella of Bory de St. Vincent, but in a subsequent publication he changed the spelling to Gallionella. This name has been most widely applied by those who have studied the organism, but the species has been referred to a considerable number of different genera of algae and of bacteria by various authors (see Buchanan, 1925).

Griffith, in 1853, pointed out that the organism was not a diatom, and could not therefore be retained in the genus Gaillonella. He created the genus Didymohelix, under the mistaken impression that the microbe is composed of two intertwining filaments. Since the genus Gaillonella has been retained for certain diatoms, and since Ehrenberg offered no reason for his change of the spelling, Buchanan accepts Didymohelix as valid. But it would seem that, whether by accident or design, the change in spelling by Ehrenberg did actually create a satisfactory generic name for this organism, which is not preoccupied and is supported not only by priority but by the more important advantage of common usage.

These vagaries in nomenclature and classification may be attributed almost entirely to the complete failure of all workers previous to Cholodny to comprehend the peculiar morphology of this bacterium. It is a stalked bacterium, the stalks being composed of ferric hydroxide deposited in colloidal form from one side of the cell. As the cell grows, and the stalks increase in length, the cell slowly rotates on the stalk, which thus becomes spirally twisted. The bacterial cell is very minute as compared with the stalk, and is easily broken off. Previous workers thus observed only the inert stalks, and based their morphologic descriptions entirely upon them. It was only by observing the morphology of the organism *in situ* upon coverslips immersed in iron-bearing waters that Cholodny was enabled to solve the riddle.

The cells are kidney- or bean-shaped, 1.2 to 1.5μ in length and 0.5 to 0.6μ in thickness. They lie upon the tips of the stalks with their long axis at right angles to that of the stalk, as in the case of *Bacterium pediculatum*. They are attached to the stalk on their concave side. They multiply by transverse fission, and, following cell division, each cell continues to spin its own stalk, so that branched stalks result. The pair resulting from a cell division may move about each other, so that the two stalks become twisted together.

The stalks are, according to Cholodny, composed entirely of ferric hydroxide, because the youngest portion adjacent to the cell stains intensively with microchemical reagents for iron, and because the whole stalk completely disappears when treated with dilute hydrochloric acid. The stalks are very brittle, easily fragmenting on handling of the material. Although Cholodny described the organism as "festsitzende," no holdfasts are mentioned.

The organism is psychrophilic, growing best in cold brooks and in the springtime. It has not been cultivated upon artificial media.

Although classed by all previous authorities with the Chlamydobacteriales, it is obvious from Cholodny's studies that this classification is false. The organism is neither filamentous nor ensheathed.

Gallionella minor

This species, discovered by Cholodny, is similar to the preceding as regards the morphology of the cells, but differs in having shorter, thicker, less twisted stalks, which often become encrusted with a deposit, forming warty outgrowths that completely mask the band-like structure of the stalk. Both species may be found side by side in the same habitat.

Hyphomicrobium vulgare

First discovered by Rullmann, this organism had apparently also been observed previously by Winogradsky and by Stutzer and Hartleb, in all cases in cultures made for the purpose of isolating nitrifying bacteria. Rullmann believed that it is a nitrifying bacterium, and named it "*Nitrobacterium formaenovae.*" It grew on all ordinary media as a short rod, but upon nitrate media developed a peculiar morphology; from the rods fine hair-like processes grew out from one pole, resembling monotrichous flagella. They were, however, not flagella, since they stained readily (though faintly) with ordinary basic dyes, and the cells were non-motile. Moreover, the fine filaments were sometimes branched, especially in liquid cultures. This branching suggested a "Streptothrix," but Rullmann, who had worked extensively with soil actinomycetes, states definitely that there is no resemblance.

Nevertheless, Hartleb and Stutzer believed that the fine filaments are mycelia, and that the bacterial cells are chlamydospores from which this mycelium sprouts. They also believed it to be a nitrifying organism, and called it the "saltpeterpilz." In a later paper Stutzer and Hartleb described the microbe more fully, and gave it the name *Hyphomicrobium vulgare.* The following summary of their description is taken from the paper by Enlows: "A nitrifying (?) organism found in soil. Related to the bacteria and to the hyphomycetes. On nitrate agar, small homogeneous rods, with usually pointed ends, 0.6 to 0.8μ by 1 to 1.5μ long. Stained with phenol fuchsin a darker central body surrounded by a clear zone may be observed. Egg-shaped forms in older cultures, which send out threads, some of which

show true branching. Multiplication also by transverse division. Found also in cement which they think was decomposing through the assistance of this organism."

The same or a similar organism was reported by Joshi as a nitrite-forming bacterium. We have not been able to see the original paper. The following summary has been given by Gibbs: "This organism was commonly found in two forms, one of which was chalky white in appearance, thread-like and branching like a mold, while the other form was shorter and had flagella at one pole. It would not grow in bouillon or on gelatin, and preferred magnesium carbonate." The chalky form might have been an actinomyces.

Gibbs noted a "very short stem-like growth" in many strains of his pure cultures of nitrobacter. Such growth is very noticeable when the preparation is stained by any method for staining flagella, but is seldom seen in the ordinary stained preparation.

Fred and Davenport observed in their studies of nitrobacter that "a great many of the mounts showed the cells with rather thick straight flagella-like attachments but never more than one to a cell. Occasionally these would have the appearance of typical flagella in that they were fragile and waved."

Although Stutzer and Hartleb, and also Winogradsky, had expressed doubt concerning the ability of the hyphomicrobium to oxidize nitrites, Prouty was apparently the first to look upon these stalked forms as contaminating and non-oxidizing. He states that: "In many instances the polar flagellum-like attachment which was noted when this organism was stained with cold carbol fuchsin, appeared to join a smaller deeply stained body to the bacterial cell. The length of this attachment varied in different cells. On many it was from 7 to 10μ long, whereas on others it was much shorter. In some preparations the organisms were clustered together with the flagella-like attachments radiating away from the center of the cluster of bacteria. In many instances the cells were unevenly stained, there being an area at one end of the cell, sometimes near the flagellum-like attachment and sometimes near the other end, which would not take the stain. These cells were generally oval and somewhat pointed at the ends."

The nature of *Hyphomicrobium vulgare* has recently been more extensively investigated by Boltjes, who has also concluded that it is not a nitrifying organism, but a common accompanying organism in cultures of nitrobacter. It appears regularly in such cultures because it is apparently widespread, can utilize nitrates as a source of nitrogen, and can grow, like the *Bacillus oligocarbophilus* of Beijerinck and van Delden, upon such organic matter as can be obtained from the air. When inoculated into inorganic media and supplied with air filtered through sulphuric acid and permanganate, no growth occurred. It grew, however, on a variety of organic media; a nitrate, sodium formate medium was especially favorable.

Boltjes definitely identified the filamentous attachments to the cells as stalks. They develop more strikingly in the media of lower nutrient value, and increase in length with age of the culture. The filaments cannot be readily seen in wet preparations observed by transmitted light, but become very obvious when dark-field illumination is used. In older cultures the stalks are definitely branched. The cells are oval in form. In young cultures they are motile, but when such motile cultures are examined by dark-field illumination, it is observed that the stalk is stiff and takes no part in propelling the organism. The stalks frequently show small knobs. It is typical that the bacteria often form stellate groups with the stalks turned outside and the knobs inside. The mode of reproduction could not be clearly observed, but Boltjes suspects that the process is different from that in "ordinary" bacteria. He noted strongly refractile bodies within the cells which were shifted slowly to the end, and believed that these were points where the newly developing cells are formed.

Summarizing the preceding statements, it would seem that *Hyphomicrobium vulgare* is a fairly definite bacterial species, widespread in distribution and capable of growing in extremely dilute media, with oval cells which tend to show some internal differentiation, and which grow upon stalks. Structures which we would interpret as holdfasts were observed by Prouty and by Boltjes; branching of the stalks was noted by Rullmann, Stutzer and Hartleb, and by Boltjes. A differentiation of the protoplasm

into deeply stained and faintly stained portions was described by Prouty and is clearly shown in the drawing accompanying the paper of Fred and Davenport; this may be related with the refractile body observed by Boltjes.

Pasteuria ramosa

In the course of his early studies on phagocytosis, Metchnikoff discovered a microörganism parasitic in the body cavity of the water fleas, *Daphnia pulex* and *Daphnia magna*, which he referred to the bacteria and named *Pasteuria ramosa*.

It appears within the body cavity of the Daphnia as globular colonies, cauliflower-like in appearance, made up of pear-shaped cells attached to each other at their tips, to form branching and rebranching lobes. At times these colonies break up into smaller ones, and continue to separate until all of the individual cells are liberated.

The cells look like little grape seeds, and in stained preparations the individual cells may be seen to be composed of three portions; an anterior rounded body, the spore, a median thickened portion, and a posterior tapering portion. The latter part serves as the attachment of the cells to each other, and is described as a stalk, although apparently continuous with the protoplasm of the cell proper.

The cells multiply by *longitudinal* binary fission, splitting lengthwise, and thus giving rise to the branched structures. The two daughter cells remain attached at their tips. The rounded bodies which Metchnikoff describes as spores, appear in his photomicrograph as deeply stained bud-like bodies resting in cup-shaped cells, but in the drawings from unstained cells they are indicated as arising within the protoplasm and being extruded with later development. The usual spore stain differentiates them from the protoplasm in the same manner as with common spore-forming bacteria. From the published photographs of stained preparations, the individual cells appear to be 4 to 5μ in length and 1 to 2μ in thickness.

Migula dismisses this organism as being obviously a

myxomycete or myxobacterium. However, Metchnikoff's description and illustrations are perfectly clear, and it is quite obvious that his organism is *not* either a slime-mold or a slime-bacterium. The longitudinal fission is a character foreign to the true bacteria as we know them. Metchnikoff ingeniously argues that fission in all planes, as in the Sarcinae, may be looked upon as a primitive character, and that in the later-developing rod-shaped organisms, some species may have retained longitudinal fission although the majority have retained only transverse fission. The single endospores are stressed by Metchnikoff as essentially bacterial characters. Unfortunately, the germination of these spores was not observed. The cells are non-motile at all stages.

Pasteuria ramosa differs markedly from the organisms which we are accustomed to consider as bacteria. It bears a strong resemblance to the blue-green algal genus, Chamaesiphon, which similarly forms pear-shaped cells attached to a substrate at the tip, and multiplies by the formation of endogenous reproductive bodies designated as "gonidia" by the algologists. We have observed several microörganisms growing upon slides immersed in Lake Alexander which show a marked resemblance to the organism of Metchnikoff, but which produce reproductive bodies apparently by budding rather than by endogenous formation. Metchnikoff clearly shows the development of spores internally. Instead of being liberated by dissolution of the cell, as in the ordinary spore-forming bacteria, they are extruded at the unattached end of the cell, a process somewhat analogous to the formation of basidiospores in the Basidiomycetes. Metchnikoff's photomicrograph shows no traces of a membrane about the spore, though such a membrane is clearly presented in his drawing.

Whether this microörganism should be considered a bacterium or not will depend upon one's definition of bacteria. In its minute size, lack of pigment, and parasitic habit of growth it resembles bacteria more than it does the blue-green algae, and it certainly cannot be included with any of the other known groups of microbes.

Bacillus flagellatus

In 1905 Mabel Jones reported a peculiar organism isolated from the city water supply and from sewage in Chicago. This bacterium grew readily on artificial culture media. It was a Gram-negative, comma-shaped organism, 1.5 to 3μ by 0.5 to 0.7μ, actively motile with a long single polar flagellum. The cells showed a marked tendency to become grouped in rosettes, with all of the flagella pointing toward an unseen center, the cells extending from the periphery, "the flagella appearing in unstained preparations of rosettes as the radial arms of a windmill to which the vanes are attached." No motility was observed in cells thus arranged in rosettes.

Nine years later Omeliansky reported finding an organism, identical in morphology with that described by Miss Jones. It grew upon plates of glycerol peptone agar which had been inoculated from a crude culture of red sulphur bacteria obtained from river water. The colonies were brownish. Slides showed the same vibrio types with single polar flagella readily visible in slides simply stained with methylene blue, and the same arrangement in rosettes. But in Omeliansky's photographs one may readily see the attachment of the so-called flagella to a common base, and small rounded expansions at the tips of the "flagella" of cells which are not in rosettes, that look like the holdfasts of some of the stalked bacteria which we have observed. Omeliansky makes no mention of motility. Subcultures failed to grow.

The strong resemblance between the organisms shown in illustrations accompanying both of the above papers and the stalked bacteria which we have observed upon immersed slides leads us to believe that the structures described as flagella were in reality stalks. This is supported by the fact that they stained so readily without any mordanting. Opposed to this view is the motility definitely recorded by Miss Jones.

Vibriothrix tonsillaris

This organism was first described by Davis and studied further by Davis and Pilot, and by Davis and Hall. It is found in the crypts of human tonsils forming small greyish or yellowish

granules. Similar granules have also been found in dental root abscesses and in sputum from cases of bronchiectasis. They have been repeatedly mistaken for granules of actinomycetes, with which, however, they are not related.

The granules are made up of central shafts of "mycelial filaments" about which radiate bacillary forms, resembling closely the fusiform bacilli of the mouth. Associated with the bacillary forms are cocci and spirilla, which, however, may be other organisms mechanically adhering to the granules.

These granules have been cultivated by Tunnicliff, and further studied in cultures by Tunnicliff and Jackson. They grow anaerobically in ascites agar, in the form of granules similar to those occurring in the tonsils. A number of coccoid and spiral forms were also found in cultures, but it is not certain that these were pure cultures.

The essential structure is a central filament, or more commonly a bundle of filaments, to which the bacteria cells are attached. Both Davis and Tunnicliff have intimated that the relationship is like that in a mold, that the central filament is mycelium and that the bacillary bodies are conidia. We suggest, however, that the filaments are *stalks* secreted by the bacteria, and that the granules are merely colonies of stalked bacteria.

This opinion was arrived at first by our observation of distinctly stalked fusiform bacilli on slides submerged in lake water. The bacillary bodies as described by Davis are indistinguishable from fusiform bacilli, both in form and in staining reactions. Tunnicliff noted that the ends of the cells in cultures were rounded or square rather than pointed, but did find the characteristic granules staining red with Giemsa stain which are present in fusiform bacilli.

The central filaments are very fine, apparently unbranched, and stain faintly as compared with the bacillary forms. They are homogeneous in structure. These characters resemble closely those of the stalks of the bacteria we have studied, not an organized protoplasmic mycelium.

This organism was referred to the genus Vibriothrix of Castellani and Chalmers by Tunnicliff and Jackson at the sug-

gestion of Castellani. The genus has been so vaguely described, however, that it might include almost any pleomorphic bacterium.

STALKED BACTERIA FROM LAKE ALEXANDER

A variety of stalked bacteria which we have observed on slides immersed in Lake Alexander are illustrated in plate 2. It will be more convenient to describe them by reference to the various figures of this plate. All of these figures have been drawn accurately from projected photomicrographs. They are illustrated in this manner to conserve space. Photomicrographs of four types are, however, presented in plate 3. Although all of the types here reported are described from Lake Alexander, we have found many of them to be ubiquitous, and anyone may readily confirm our observations by examining slides immersed for a few days in stagnant water, or even in running tap water.

Figure 1 shows a stalked vibrio which appears very similar to the illustrations published by Miss Jones and by Omeliansky. It is a trifle larger than the dimensions given by Miss Jones. The cells are always distinctly curved, with rounded ends. Multiplication occurs by transverse binary fission. The stalks are very slender, and often show a distinct button-like expansion at the end, which we consider to be a holdfast. It is probable that the outermost cell is set free after cell division, and either swims or floats away until a new substrate is encountered, when it proceeds to secrete a stalk. All examples studied by Gram's stain have failed to retain the stain.

This is probably the most common type encountered. It has been found both in the open lake and in the shallow, weed-choked bays, and at all depths up to 8 meters. It is, however, a little more abundant in the shallower waters. It has been observed at all seasons except when the lake was frozen, at which time no observations have been made.

Figure 2 shows a small, straight rod form, with short, slender stalks and very prominent, button-like holdfasts. It is Gram-negative, and multiplies by transverse binary fission. It is quite rare, and has been found on only a few slides from shallow water. When it does occur, however, large areas of the slide are covered by the rather widely scattered organisms.

Figure 3 illustrates a large, relatively straight rod form with rounded ends. It is Gram-negative. It is also relatively rare, occurring usually in groups of three or four individuals. Division has not been observed.

Figures 4, 5, and 6 show fusiform types. These are abundant and vary considerably in size. We are of the impression that they tend to group about three modal sizes, as illustrated, and that these represent three distinct species, because they often occur in rather extensive microcolonies in which all of the cells are of nearly the same size.

All of the fusiform types which we have observed by Gram's stain were negative. Multiplication is by transverse binary fission. The cells are pointed at both ends. The protoplasm stains more deeply in the center of the cell, and merges into the slender stalk. We have not yet obtained satisfactory Giemsa stains of these organisms, so we cannot say for certain whether they exhibit granules of the type shown by the fusiform bacilli of the human mouth. The stalks are slender in the smaller species, rather coarse in the largest one. Holdfasts have been observed in all.

After cell division the outermost cell develops a long slender tip, and we have in a few instances found a pair of cells with a stalk and holdfast at both extremities of the pair. We believe that in these species the outermost cell develops its stalk and becomes anchored to the substrate before the two are finally separated, in this way extending rapidly over the substrate. This view is supported by finding the fusiform types commonly in very extensive microcolonies, extending over many oil immersion fields.

The fusiform species have been found frequently in all of the lake habitats which we have investigated, and in the open lake at all depths up to 13 meters.

Figure 7 shows a puzzling form, not very frequent in occurrence, but rather widely distributed. The cells are swollen, irregular in form, and often triangular. The larger forms stain faintly in the center, as though they were ballooned by a vacuole. We are inclined to the opinion that they are involution forms of the vibrio shown in figure 1. But this opinion is opposed by finding

them in one instance fairly abundant on a slide which was immersed in the lake for only twenty-four hours.

The species considered so far all show a certain degree of resemblance. They are all essentially bacillary in form, and show definite transverse fission (save in the types shown in figure 3, of which only a small number of individuals have been examined; and in figure 7, which are probably involuntionary). While occurring in extensive microcolonies, they do not tend to form clusters, and the stalks are not branched. Although both Miss Jones and Omeliansky observed radiating clusters in their cultures, we are of the opinion that the organisms just described are very similar to those reported by these authors, and that the formation of radiating clusters may be a peculiarity of artificial cultures. When growing close together on agar, they may well become attached to each other rather than to the agar. In any case, our stalked vibrio (figure 1, plate 2) bears a striking resemblance to the organism illustrated by Omeliansky (figure 5, plate 1) which he named *Bacillus flagellatus*.

The remaining forms which we have observed differ markedly from the above mentioned types, and resemble in many respects the organism described by Metchnikoff as *Pasteuria ramosa*.

Figure 8 represents a fairly common water organism. It is a short, plump rod, not stalked, but showing in common with the stalked bacteria a polar differentiation of the cells. There is a base and a tip. At the base an amorphous material, probably gum, is secreted to fasten the cells to the substrate. This material often extends from the cell in a fan-like manner. The Gram reaction is variable, both positive and negative individuals appearing in the same field. Multiplication apparently is entirely by budding, or by a process intermediary between budding and fission. The buds present the form of the parent cell, but are smaller both in length and in thickness.

Figure 9 shows a somewhat similar organism without a definite holdfast. The cells are swollen at one end, and stain deeply at this end, faintly at the pointed end which is probably attached to the glass in some manner. Occasionally two cells are arranged in a V form, possibly indicating recent longitudinal fission, but this

is not certain. Multiplication by buds is at least the more frequent, if not the sole, mode of multipliction. Young buds are spherical, but become oval or even rod-shaped before they are liberated.

Figure 10 illustrates a species which is more common than the two preceding, and which differs from them in occurring distinctly in rosettes. The cells are elongated and piriform in shape, and are attached to each other at their tips. They usually stain solidly. Often a mass of cementing material may be seen about the common base of a rosette, and occasional isolated cells show a distinct short stalk and holdfast. The rosettes very obviously arise by longitudinal fission. In addition, reproduction by budding is commonly observed. The buds are oval to rod-shaped in form. Save for this last character, the organism is almost identical in morphology with the *Pasteuria ramosa* of Metchnikoff.

This species has been found with equal abundance in all of the habitats studied in Lake Alexander. In the open lake it occurs at all depths up to 13 meters.

Figure 11 shows a small coccoid form which is not definitely a stalked bacterium, yet shows some resemblances to the type shown in figure 10, and may possibly be a growth form of the type illustrated in figure 14. The cells occur in clusters cemented together by amorphous material, and tend to be arranged in rosettes. The outermost cells often show minute spherical buds, and this is the only mode of multiplication that we have observed. These forms are not abundant.

Figure 12 represents another organism of very doubtful nature, which is offered here merely for comparison with the preceding. It is a large spherical organism multiplying by budding. It may possibly be a small yeast, but it would be surprising to find yeasts growing attached to a firm substrate in water.

Figure 13 presents two examples of a group of microbes which show a variety of forms, and which will require further study before their specific characters may be delineated. They occur as single globular, oval or pear-shaped cells upon a stalk which is fastened to a substrate. There is an oval type whose cells are often vacuolated, which is especially common. Multiplication is apparently entirely by budding.

Figure 14 illustrates one of the most striking of the stalked microörganisms which we have encountered. The cells are globular in form, attached to long slender stalks which radiate from a common center. Multiplication is by budding, and the buds are also globular. The smaller cells stain solidly, but the larger cells which are budding show a differentiation of the protoplasm, the outer part staining deeply while that part of the cell to which the stalk is attached stains more faintly. As many as 8 stalks have been observed attached to a common holdfast. Usually they are attached directly to the glass, occasionally to algae or other organisms or to some amorphous débris. The young, solid-staining cells are Gram-positive, but budding individuals are usually Gram-negative.

We believe that the characteristic growth of this organism in whorls may be best explained by assuming that when the buds germinate they first undergo a multiple fission, perhaps producing clusters of cells as shown in figure 11, and that then, from these clusters, the individual cells secrete stalks, which thus radiate from a common holdfast.

This organism is distinctly psychrophilic. It has been found only in the open lake, where temperatures do not exceed 23°C., never closer to shore than the 2-meter contour. It has been found constantly in several different stations in the open lake, at all depths up to 13 meters. It occurs more abundantly in the fall months than in the summer.

Figure 15 presents a type which differs from the preceding only in the thick, tapering stalks. We cannot determine whether this is a separate species. In some clusters both thick stalks and slender stalks occur. Both types of stalks may be formed by the same species, or a slender-stalked species may become attached to a thick-stalked one. Both types may be found on the same slide.

Figures 16 and 17 illustrate another species resembling the preceding. The cells are larger and pear-shaped. The same peculiar difference in the staining of budding and non-budding cells is observed. Mature buds are themselves pear-shaped, and may also show a differential staining. This organism is also

peculiar in that we have never observed it attached directly to the glass. It is always attached to a mass of amorphous débris adherent to the glass.

This pear-shaped organism is not nearly so abundant as the globular form illustrated in figure 14. It is also found only in the open lake, and has been observed at depths up to 7 meters. Curiously, it was much more abundant in 1934 than in 1933.

In all of the microörganisms illustrated in figures 8 to 17 inclusive we have referred to multiplication by budding as a common and striking character. Concerning the nature of these buds we are still a little in doubt, because they resemble to some extent the bodies described by Metchnikoff as spores in *Pasteuria ramosa*. We have attempted to demonstrate a membrane surrounding the bud by various staining methods, especially by Fontana's silver impregnation, without success. Conversely, by the use of Congo-red negative staining we have clearly demonstrated that there is no membrane; the stain penetrates readily between the bud and the parent cell, leaving only a small band of attachment. Moreover, we see all stages of development in the buds, from minute globular bodies to masses nearly as large as the parent cell. We are forced to conclude therefore that either Metchnikoff was dealing with an entirely different sort of a microbe, or that his observations were erroneous. The strong resemblance between the organism illustrated in our figure 10 and Metchnikoff's *Pasteuria ramosa* inclines us to believe that we are studying microbes of the same general character.

There is a strong resemblance between the organisms illustrated in figures 13 to 17 (plate 2) and the *Hyphomicrobium vulgare* of Stutzer and Hartleb. In both, there is a tendency for the cells to assume a globular or ovoid form, and to show a differential staining in the protoplasm. In both, there is a tendency to dichotomous branching of the stalks, which may be explained only upon the basis of longitudinal fisssion. The photomicrograph which Rullmann published might well pass for an illustration of the type which we have illustrated in figure 14, save for the absence of buds. In fact, the complete absence of any mention of budding by the various authors who have studied

hyphomicrobium is all that prevents us from concluding that our organisms are identical or closely related. Until the mode of reproduction of hyphomicrobium has been determined, we shall be unable to state definitely to what degree it is related to the forms which we have described.

Figure 18 is another organism of very doubtful nature, in fact we are not certain that it is a microörganism. It is offered here merely for record, since it may possibly with further study prove to be a stalked bacterium.

The material appears in tangled masses much resembling windrows of hay. The portion illustrated is less dense than usual. These masses are composed of fine, faintly-stained filaments, which do not appear to branch. At either extremity of the filament is a deeply stained spherical body, one of which usually appears a little larger than the other. It is possible that one is a cell and the other a holdfast, but both are close to the limits of resolution of the microscope, and nothing may be said with certainty.

CULTIVATION

No extensive experiments have been carried on with a view to the isolation of these organisms in pure culture and their study by standard methods, since we have been engaged primarily in a study of the bacteria by purely microscopic methods in their natural habitat. A few crude cultures have, however, been obtained.

Samples of water from Lake Alexander were collected in the summer of 1934 and brought to the laboratory in Minneapolis. They were inoculated into a variety of liquid media. A growth of stalked bacteria was obtained in two of these, one a mineral solution containing precipitated cellulose, with ammonium salts as the nitrogen source; the other a solution of $MgSO_4$ and K_2HPO_4 in tap water, to which bits of exoskeleton of marine crabs were added. The types shown in figures 1, 4, and 13 (plate 2) appeared in both of these media after 48 hours incubation, but together with a great many other types of bacteria, especially vibrios. It was possible to carry them through separate transfers,

and some are still growing after five months. But it has been impossible to obtain a growth upon agar, and therefore we have obtained no pure cultures as yet. Further attempts are in progress. These cultures were incubated at room temperature in the dark.

TAXONOMY

A consideration of the classification of these microbes at once raises the question whether they may be considered as bacteria or not. Some of them are obviously bacteria, but others, such as the pear-shaped and globular forms multiplying by longitudinal fission and by budding, seem at first glance to be far removed from the organisms ordinarily thought of as bacteria.

We offer as essentially bacterial characters, the following: *1. Size.* The largest observed is but a little over 2 μ in diameter. They might be very small forms of algae or protozoa, but in that case one would expect to observe forms intermediary between these minute bacteria-like bodies and the larger microbic species. This has not been observed. *2. Structure.* While in some of the larger species there has been observed a differentiation of the protoplasm into a deeply stained and faintly stained portion, this is in general true only of budding cells, and is not a constant characteristic. There is, therefore, no differentiation of the protoplasm, such as would be expected in cells of algae or protozoa. Nothing resembling a nucleus or central body has been observed. *3. Nutrition.* The fact that many of the forms described have been uniformly distributed in depths up to 13 meters indicates that they are not photosynthetic. The algae which grow upon the same slides show a marked gradation from the surface to the bottom, obviously correlated with light penetration, and are relatively rare at depths below 3 or 4 meters. Further, the fact that some of these forms have been grown in the dark in crude cultures containing organic matter indicates that they are not photosynthetic, but suggests that they are heterotrophic. *Gallionella ferruginea* is usually considered autotrophic, though this is denied by some authorities. It is obvious that none of these stalked bacteria are capable of ingesting solid food like the

protozoa. It seems highly probable, therefore, that as a group they are heterotrophic, i.e., saprophytes. Metchnikoff's organism was parasitic.

The best argument for including these microörganisms with the bacteria is the fact that they cannot be included in any other group. Their minute size, structureless cells, and saprophytic or parasitic modes of life indicate that they belong with the bacteria rather than with the algae or protozoa, the only other possible places for them. Our conceptions of the limitations of the class "Schizomycetes" are still rather vague. There is nothing in Bergey's definition of the class which would exclude the organisms here considered.

Assuming that they may be properly included with the bacteria, it remains to determine how they should be grouped within this class. The taxonomic value of morphologic characters increases with decreasing diversity of form of the organisms to be classified. It seems to us that with microbes so slightly diversified as the bacteria, the occurrence of structures so unusual as stalks is sufficient to justify the recognition of such bacteria as a distinct order, certainly sufficient to distinguish them from the Eubacteriales. They are obviously not actinomycetes or spirochaetes. We have already pointed out how they differ from the myxobacteria. They resemble the Chlamydobacteriales in presenting an axial differentiation of the cells into basal and apical portions, and in their aquatic habitat, and in the formation of holdfasts; but differ in failing to form filamentous chains of cells, and sheaths. While one species may possibly be concerned with the oxidation of sulphur, this is not true of the group as a whole.

It may be questioned whether the grouping of all stalked bacteria into a separate order is a "natural" arrangement, i.e., whether these organisms are actually phylogenetically related. Concerning this, we do not have enough information to venture an opinion, but the same is equally true of the other orders of bacteria as now defined. Such a classification is, however, of some practical value. It brings together in some order a variety of little known forms whose descriptions are widely scattered in the literature, and which so far have had no place in the recognized classifications of bacteria.

We therefore propose the creation of a new order, defined as follows:

Caulobacteriales. Bacteria growing characteristically upon stalks. The cells are asymmetrical in that gum, ferric hydroxide, or other material, is secreted from one side or from one end to form the stalk. Multiplying typically by transverse binary fission; in one family by budding and longitudinal fission. In some species stalks may be very short or absent, the cells connected directly to the substrate or to each other by holdfasts. Cells occur singly or in pairs, never in chains or filaments; not ensheathed. Typically aquatic in habitat; some may be parasitic in animals.

The order is naturally subdivided into four divisions by major differences in morphology. We recognize these divisions as families.

I. **Nevskiaceae.** Stalked bacteria, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks lobose, dichotomously branched, composed of gum. Multiplication of cells by transverse binary fission. Growing in zoögloea-like masses in water or in sugar vats.

One genus, *Nevskia* Famintzin. Type species, *Nevskia ramosa* Famintzin. A second species is *Nevskia pediculata* (nov comb.) (Koch and Hosaeus). H. and J.

II. **Gallionellaceae.** Stalked bacteria, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks are slender, twisted bands, dichotomously branched, composed of ferric hydroxide. Multiplication of cells by transverse binary fission. Growing in iron-bearing waters.

One genus, *Gallionella* Ehrenberg. Type species, *Gallionella ferruginea* Ehrenberg (more accurately described by Cholodny). A second species is *Gallionella minor* Cholodny.

III. **Caulobacteriaceae.** Stalked bacteria, the long axis of the elongated cells coinciding with the axis of the stalk. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, unbranched. Multiplication of cells by transervse-binary fission. The outermost cell of a pair may form a stalk before cell division is complete. Periphytic, growing upon submerged surfaces.

One genus, *Caulobacter* (nov. gen.).

If it could be established beyond question that the organism described by Omeliansky as *Bacillus flagellatus* was actually stalked rather than flagellated, this could readily be taken as the type species. Since this cannot be done, we propose as the type species the organism illustrated in figure 1, plate 2, as:

Caulobacter vibrioides (n.s.). Cells elongated, curved, with rounded ends, 0.7 to 1.2 × 2–5 microns.

The types illustrated in figures 2 to 7 inclusive (plate 2) will also fall in this genus, but specific names will not be given until they are studied further.

IV. **Pasteuriaceae.**[2] Stalked bacteria, the long axis of elongated cells coinciding with the axis of the stalk. Stalks may be very short, in some species lacking, but when present are usually very fine and at times arranged in whorls attached to a common holdfast. Cells multiplying by longitudinal fission or by budding, or both, spherical or pear-shaped in most species. Mostly periphytic, one species parasitic.

Genus 1. *Pasteuria* Metchnikoff. Stalks short or absent, the cells attached directly to the substrate or to each other, often with distinct holdfasts. Type species, *Pasteuria ramosa* Metchnikoff.

The organisms which we have illustrated in figures 8 to 10 inclusive (plate 2) will fall in this genus. We withhold specific names until they have been studied further.

Genus 2. *Blastocaulis* (nov. gen.). Stalks long and slender, often arising in whorls from a common holdfast.

We take as the type species the organism illustrated in figure 14 (plate 2), which we name:

Blastocaulis sphaerica (n.s.). Cells spherical, multiplying characteristically by budding.

The organisms shown in figures 13, 15, 16, and 17 also belong in this genus, but specific names are withheld until further studies have been completed.

[2] A family of "Pasteuriacées" was proposed by Laurent to include the organism of Metchnikoff and the root-nodule bacteria of legumes. It is now, however, obvious that the branched bacteroids of *Rhizobium leguminosarum* have nothing in common with these stalked bacteria. We retain the family name, but redefine it.

The proposed classification takes care of the well-defined types. It does not include the organisms illustrated in our figures 7, 11, 12, and 18 (plate 2) nor the *Vibriothrix tonsillaris* Tunnicliff and Jackson, which are as yet too poorly understood for classification. We believe that *Hyphomicrobium vulgare* will eventually prove to be identical with one of the species of our genus Blastocaulis, but we cannot definitely place it in our classification until its mode of reproduction has been discovered.

SUMMARY

A study of periphytic bacteria upon glass slides immersed in freshwater habitats shows the general occurrence of a group of bacteria hitherto almost unknown, which secrete stalks by which they are attached to a firm substrate. A search of the older bacteriological literature has shown that similar types have been observed before, and has also revealed certain other kinds of stalked bacteria which we have not observed. It is proposed to include all of these stalked bacteria in a new order of Schizomycetes, the **Caulobacteriales**. It is proposed to subdivide this order into four families and five genera according to the following key:

A. Long axis of cell transversed to long axis of stalk; stalks dichotomously branched.
  I. Stalks lobose, composed of gum, forming zoögloea-like colonies.
    Family **Nevskiaceae**
    Genus *Nevskia*
  II. Stalks are twisted bands of ferric hydroxide
    Family **Gallionellaceae**
    Genus *Gallionella*
B. Long axis of cell coincides with axis of stalk.
  I. Reproducing by transverse fission, stalks unbranched
    Family **Caulobacteriaceae**
    Genus **Caulobacter**
  II. Reproducing by longitudinal fission and by budding; stalks often branched in whorls.
    Family Pasteuriaceae
    a. Stalks very short or lacking, cells sessile
      Genus *Pasteuria*
    b. Stalks long and slender
      Genus **Blastocaulis**

The scientific names in this summary, which are set in boldfaced type are new.

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PLATES

PLATE 1

Stalked Bacteria from the Literature

All figures have been redrawn from the original publications

Fig. 1. *Nevskia ramosa* Famintzin. (*a*) Zoögloeal masses, showing bacteria at the tips of the stalks. (*b*) Bacterial cells showing prominent fat globules.

Fig. 2. *Bacterium pediculatum* Koch and Hosaeus.

Fig. 3. *Pasteuria ramosa* Metchnikoff. (*a*) From photomicrographs of cells and clusters of cells, stained. (*b*) From photomicrograph of a cell with a spore. (*c*) From drawings of colonies, unstained. (*d*) Vegetative cells, unstained. (*e*) To *l*, stages in spore formation and liberation.

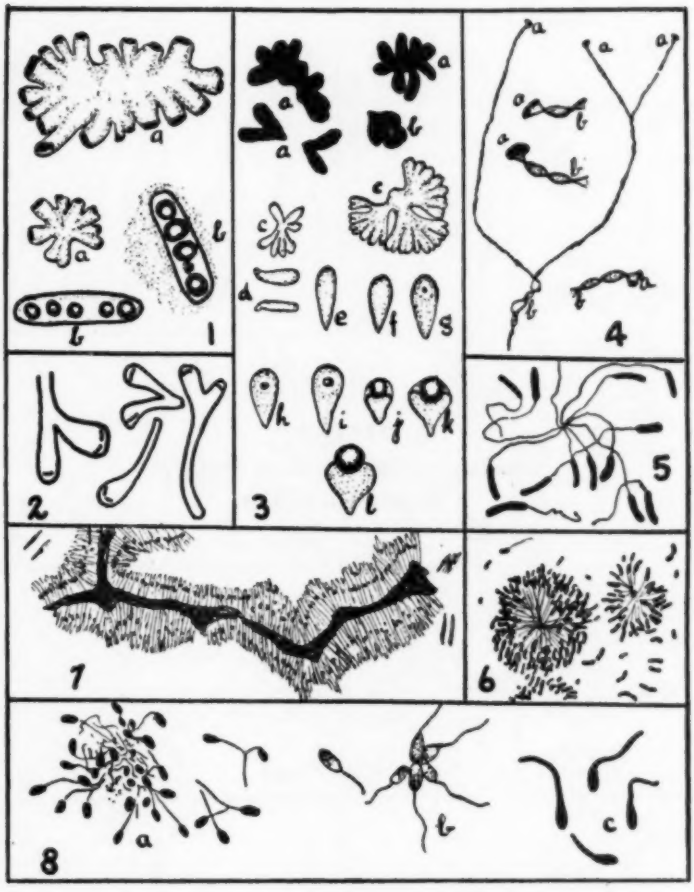
Fig. 4. *Gallionella ferruginea* Ehrenberg, after Cholodny. (*a*) Bacterial cells. (*b*) Stalks of ferric hydroxide.

Fig. 5. *Bacillus flagellatus* Omelianski.

Fig. 6. The organism of Mabel Jones.

Fig. 7. *Vibriothrix tonsillaris*, Tunnicliff and Jackson.

Fig. 8. *Hyphomicrobium vulgare* Stutzer and Hartleb. (*a*) After Rullmann. (*b*) After Fred and Davenport. (*c*) After Boltjes; Zettnow's stain.



(Arthur T. Henrici and Delia E. Johnson: Studies of Freshwater Bacteria)

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PLATE 2

STALKED BACTERIA FROM LAKE ALEXANDER

Explained in the text

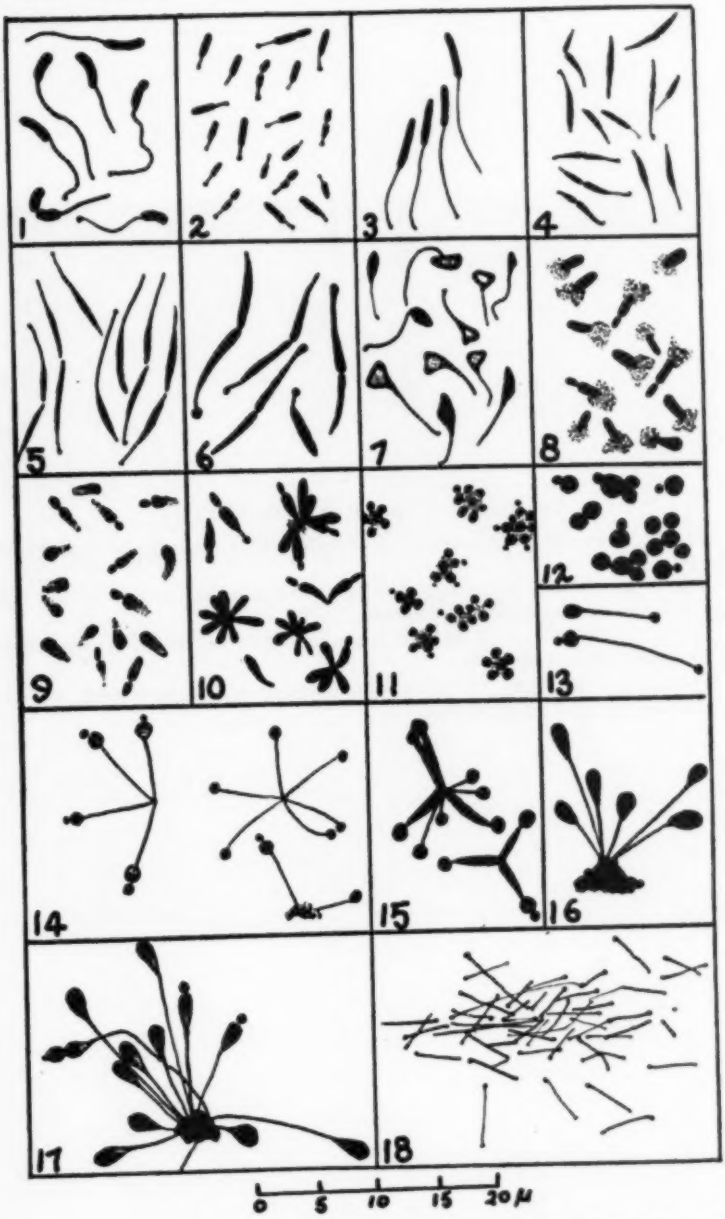

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PLATE 3

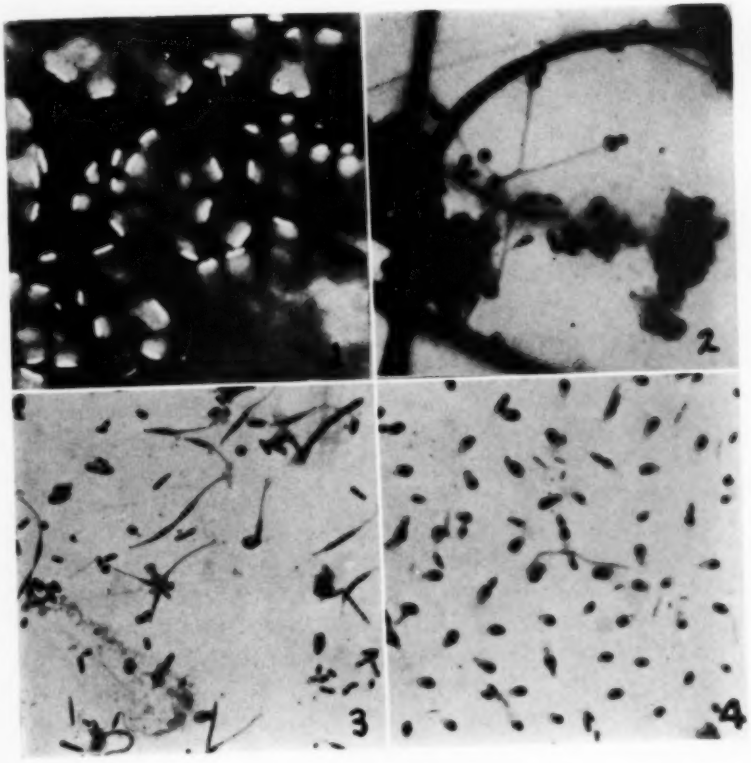
Photomicrographs of Stalked Bacteria

Fig. 1. *Nevskia sp.*, cultivated in water with sulphide; negatively stained with Congo red.

Fig. 2. *Blastocaulis sphaerica*, showing branched stalks and budding. The coarse filaments are *Cladothrix dichotoma*.

Fig. 3. *Caulobacter sp.* This is the larger fusiform type; holdfasts may be seen at the tips of some of the stalks.

Fig. 4. *Pasteuria sp.* The differential staining of the protoplast, and reproduction by budding are shown.



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A STUDY OF THE CORYNEBACTERIA ASSOCIATED WITH DISEASES OF DOMESTIC ANIMALS[1]

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Corynebacteria found in human tissue have been rather completely studied and classified. This has been due largely to the important relationship many of them have had with *Corynebacterium diphtheriae*. The Corynebacteria of animal origin, however, have not been studied or classified adequately. Diphtheroid bacteria are the causes of acute and chronic diseases of animals, and some species have been described which are of great importance to animal health. Space does not permit a complete review of the literature of these organisms, but a few important contributions should be mentioned.[2]

Corynebacterium pyogenes is probably the most important, since it is the organism found in more types of lesions than any of the others. This organism has been isolated from suppurative pneumonia and arthritis in swine and chronic suppurative conditions in cattle and sheep, particularly mastitis. From the time of the original descriptions of the organism by Lucet (1893), Grips (1902), Kunneman (1903), and Glage (1903), the organism has been isolated from a variety of tissues and animals by numerous investigators. Most of the work was done in European countries, particularly Germany. Brown and Orcutt (1920) in the United States studied it in detail. The most recent and complete study of *C. pyogenes* was by Rolle (1929). Ray (1928) recorded finding

[1] A thesis presented as partial fulfilment for the degree of Doctor of Philosophy at Iowa State College in 1933.

[2] The detailed literature concerning the Corynebacteria associated with animal diseases may be obtained by consulting the original thesis on file at the Iowa State College Library, Ames, Iowa.

a diphtheroid associated with pulmonary edema of swine, which, because of its location and species of animal infected, may have been *C. pyogenes*.

Since the original studies of Nocard (1896) and Preisz (1894), *Corynebacterium pseudotuberculosis* has been known to cause caseous lymphadenitis in sheep and ulcerative lymphangitis in horses. Nörgaard and Mohler (1899) described the occurrence of caseous lymphadenitis in the United States. This organism also has been found in suppurative conditions in cattle. Daines and Austin (1931) found it in skin lesions of tuberculin-reacting cattle.

Corynebacterium renalis was described in detail by Ernst (1905, 1906) in his study of the cause of pyelonephritis of cattle. Jones and Little (1925) also found this organism associated with pyelonephritis of cattle.

Magnusson (1923) was the first to describe *Corynebacterium equi* after isolating it from cases of pneumonia of colts. Dimock and Edwards (1931) added to the knowledge of this organism when they described cultures which had been isolated from foals.

There has been no consistency in the nomenclature and classification of the Corynebacteria of animal origin by various authors, and some species have been entirely overlooked.

Eberson (1918) probably was the first to place *C. pseudotuberculosis* and *C. pyogenes* under the generic name Corynebacterium.

Buchanan and Murray (1922) considered only one diphtheroid, *C. pseudotuberculosis*, of any consequence in animal disease. *C. pyogenes* was placed in the genus Hemophilus and assigned the name *Hemophilus pyogenes*.

Minett (1922) studied 53 strains of Corynebacteria from various sources, and on the basis of morphology, biology and immunity (agglutination) placed them in the following groups: I. Preisz-Nocard group; II. Wound diphtheroids; III. Skin diphtheroids; IV. Conjunctival diphtheroids, (a) equine, (b) bovine, (c) human; V. Nasal diphtheroids (horses); VI. Urine diphtheroids (a) equine, (b) bovine, (c) human; VII. *Bacillus pyogenes*; VIII. *Bacillus lipolyticus*; IX. *Bacillus enzymicus*; X. Throat diphtheroids

(human). The article did not give any of the characteristics of the above groups.

Andrewes *et al.* (1923) included *C. pseudotuberculosis-ovis* and *C. pseudotuberculosis-murium* in a group which they titled "Special Diphtheroid Series." They observed that the latter organism would find a place in their group 7 with a number of human strains which they studied. *C. pseudotuberculosis-ovis* was not placed in any of their groups.

Bergey *et al.* (1934) placed the diphtheroid organisms from animals in a group composed of short, thick, straight, barred and occasionally club-shaped rods. There appear to be many discrepancies in the descriptions of these organisms. *C. pseudotuberculosis* is the name given to a Gram-negative rod found in rodents. One of the cardinal characteristics of the Corynebacteria is their positive reaction to the Gram stain. The above organism is usually considered a Pasteurella. Bergey omits *C. pyogenes*. The well established *C. pseudotuberculosis*, which causes caseous lymphadenitis in sheep and ulcerative lymphangitis in horses is given the name *C. ovis*. *C. bovis* is given the credit for causing pyelonephritis in cattle while the organism *C. renale*, which has been considered to be the etiologic agent, has been relegated to a secondary place.

Thomson and Thomson (1929) proposed five groups of Corynebacteria. *C. pseudotuberculosis-murium* was placed in group I which was composed of non-chromogenic, profuse-growing, aerobic and facultative anaerobic bacteria. *C. pseudotuberculosis-bovis* was allotted to group III which included the non-chromogenic delicate growers, aerobic or anaerobic. *C. equi* was placed in group V, the chromogenic, profuse growers, aerobic or facultative. *C. pyogenes* was not classified. *C. renalis* was not mentioned.

Kelser (1933) considered three Corynebacteria of importance in animal diseases. *C. pseudotuberculosis*, *C. pseudotuberculosis-rodentium* and *C. pyogenes*. He agreed with Bergey in placing a Gram-negative organism with the Corynebacteria. He placed *C. renalis* with *C. pyogenes*. These two organisms are quite different, as will be shown in this paper. The descriptions of

C. renalis by Enderlen, Ernst and Jones and Little do not suggest *C. pyogenes* described by Poels, Brown and Orcutt.

Ford (1927) gave *C. pyogenes* and *C. renalis* specific recognition but included *C. pseudotuberculosis-ovis* in a group of bacteria which cause pseudotuberculosis.

Topley and Wilson (1929) included *C. renalis* with *C. pyogenes*. They recognized *C. pseudotuberculosis-ovis*.

Gaiger and Davies (1932) mentioned *C. pyogenes* and *C. bovis* as the Corynebacteria which cause animal diseases.

SOURCES OF CULTURES

No attempt was made in this study to isolate all possible diphtheroid bacteria from animal tissues. It is presumed that many exist as saprophytes, as do many which are found in human tissue; consequently it is probable that Corynebacteria exist which may not show the characteristics of those included in this study. An attempt was made to secure all strains of diphtheroids which have any etiologic relationship with animal disease. Representative types were obtained from other laboratories and some were isolated by the author. It is believed that the cultures isolated by the author caused the lesions from which they came.

The animal diphtheroids which have been studied sufficiently to have received specific designation are as follows:

Corynebacterium pyogenes
Corynebacterium pseudotuberculosis
Corynebacterium renalis
Corynebacterium equi

Most of the cultures which are described in this study belong to the above four species. The history of each culture is given in table 1.

STAINING METHODS

Smears for staining were made from cultures twenty-four to forty-eight hours old. Various Gram staining methods were used. The one which gave the most satisfactory results was the method described by Kolmer and Boerner (1931), in which Weigert's gentian-violet solution and acetone were used. The Ziehl-

TABLE 1

History of cultures

CULTURE	HOST ORIGIN	LESION	FROM WHOM OBTAINED
		Type I. *Corynebacterium pyogenes*	
8664	Bovine	Purulent metritis	Isolated by author
8986	Bovine	Suppurative mastitis	Isolated by author
8987	Bovine	Suppurative arthritis	Isolated by author
4823	Bovine	Pneumonia	American Type Culture collection
9292	Ovine	Suppurative mastitis	Isolated by author
		Type II. *Corynebacterium pseudotuberculosis*	
63	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
163	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
167	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
4-C-29	Bovine	Tuberculosis ? of skin	Daines and Austin—Utah
12-C-29	Bovine	Tuberculosis ? of skin	Daines and Austin—Utah
15-C-30	Bovine	Tuberculosis ? of skin	Daines and Austin—Utah
18-O-31	Bovine	Tuberculosis ? of skin	Daines and Austin—Utah
27-C-29	Bovine	Tuberculosis ? of skin	Daines and Austin—Utah
8728	Bovine	Tuberculosis ? of skin	O. G. Larson—Utah
8772	Bovine	Tuberculosis ? of skin	O. G. Larson—Utah
9126	Bovine	Purulent vulvitis	Isolated by author
809			American Type Culture collection
		Type III. *Corynebacterium renalis*	
111	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
174	Bovine	Pyelonephritis	S. H. McNutt—Iowa
1310	Bovine	Pyelonephritis	Little and Jones—New Jersey
9064	Bovine	Pyelonephritis	Isolated by author
34	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
112	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
149	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
152	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
182	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
173	Bovine	Pyelonephritis	S. H. McNutt—Iowa
169	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
74	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
8592	Bovine	Pyelonephritis	Isolated by author
8713	Bovine	Pyelonephritis	Isolated by author
8714	Bovine	Pyelonephritis	Isolated by author
W-2	Bovine	Purulent cervicitis	S. H. McNutt—Iowa

TABLE 1—*Concluded*

CULTURE	HOST ORIGIN	LESION	FROM WHOM OBTAINED
Type III. *Corynebacterium renalis*—Concluded			
183	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
164	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
165	Bovine	Purulent cervicitis	S.H. McNutt—Iowa
8124	Equine	Pus in semen	Isolated by author
Type IV. *Corynebacterium equi*			
15655	Equine	Pneumonia	Dimock and Edwards—Kentucky
Type V. Non-chromogenic—viscid			
175	Bovine	Mastitis—milk	S. H. McNutt—Iowa
176	Bovine	Mastitis—milk	S. H. McNutt—Iowa
Type VI. Non-chromogenic—profuse growers—moist			
181	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
184	Bovine	Purulent cervicitis	S. H. McNutt—Iowa
Type VII. Orange colored variants			
112a	Variant of culture 112		Isolated by author
184a	Variant of culture 184		Isolated by author
27-C-29a	Variant of culture 27-C-29		Isolated by author
95	American Type Culture collection		
8774	American Type Culture collection		
Corynebacterium diphtheriae—Park Strain 8			
296	American Type Culture collection		

Neelsen acid-fast stain was used. Cultures grown on modified Petroff's medium and in milk were stained by the Ziehl-Neelsen method on the second, fifth, tenth and thirtieth days. Albert's stain was used to demonstrate metachromatic granules. Cultures for this stain were grown on Loeffler's serum because this medium apparently caused the formation of granules more readily than any other.

CULTURE MEDIA AND TESTS

Liver-infusion serum agar was used as the stock medium for this study. It was found that a liver-infusion broth base was

more suitable than lean meat or heart-infusion broth. Not only was growth more abundant on this medium, but chromogenesis was more marked; in fact, a few cultures produced pigment only on this medium. To prepare this medium, two per cent granulated agar was added to liver-infusion broth. After sterilization in the autoclave and cooling to 45°C., bovine serum which was sterilized by filtration, was added to the agar making 10 per cent of the total volume. The reaction of the medium was pH 6.6. This medium was found to be suitable for the isolation of the cultures obtained by the author and it produced suitable growth of all the cultures which were studied.

Various types of solid media have been described for the isolation of the diphtheria bacillus and related microörganisms. Douglas (1922) described a medium composed of nutrient agar, potassium tellurite and sterile trypsinized serum. He found that colonies of Corynebacteria had a distinctive black color on this medium. In this study all the cultures grew with colonies of various shades of blackness, slate to jet black. The use of potassium tellurite in medium as a diagnostic agent for diphtheroids may lead to confusion. King and Davis (1914) studied the use of this salt as an indicator of microbial life. They described the macroscopical and microscopical appearance of most of the common species of pathogenic bacteria. It was found that all of the more common microörganisms reacted with potassium tellurite forming characteristic black compounds. The reaction was probably due to a reduction of the tellurite. They found a dilution of 1:50,000 of the salt seemed to be most suitable as a general microbic indicator. The author found that many of the common bacteria produced black colonies on Douglas medium. Allison and Ayling (1929) described an improved medium for isolating *C. diphtheriae*. They added copper sulphate to trypsinized-serum tellurite agar and found this medium inhibited the growth of streptococci and staphylococci, which were the organisms commonly found in the throat of man. The writer found this medium to be unsatisfactory for the cultures he studied. A very heavy seeding was necessary to get any growth of many cultures and the growth of *C. pyogenes* was entirely inhibited.

An excellent medium for the isolation of *C. pyogenes* was Loeffler's medium to which was added 1 cc. of 1 per cent potassium tellurite to each 100 cc. of medium. This medium was particularly suitable for *C. pyogenes* because the serum was liquefied and the tiny black colonies contrasted with the white color of the medium could be seen in the liquefied depressions. (See fig. 1.)

Hiss serum water, pH 7.0, was used for the fluid medium. For the fermentation reactions 1 per cent of the carbohydrate to be tested was added to the medium. Andrade's indicator was used to detect acid formation. Readings were made after 1, 2, 3, 5, 7, 10, 15, 20 and 30 days. When acid was formed in any of the tubes, agar slants were inoculated to determine the purity of the culture. Inoculations to determine growth were conducted on the tenth day on all cultures.

Litmus skim-milk was used to demonstrate the action of the microörganisms on milk.

The action of the various cultures on solidified serum was determined by growth on Loeffler's serum. Cow, instead of horse, serum was used.

Gelatin liquefaction was studied by growing the cultures in serum gelatin. Fifteen per cent granulated gelatin was added to liver-infusion broth to which serum, 10 per cent, was added. The cultures were incubated at 37°C. and observed every day for ten days. They were placed in a refrigerator for one hour each day in order to detect liquefaction.

Hemolysis was detected by growing the cultures in blood agar. The medium was prepared by adding sterile defibrinated rabbit's blood to a 2-per-cent heart-infusion agar, pH 7.2. Tubes were inoculated when cooled to 45°C. and agitated gently to insure an even distribution of the bacteria in the medium. The medium was poured into Petri dishes in a thin layer. The final reading under 100 magnification was made on the fourth day.

Hydrogen sulphide production was ascertained by growing on lead acetate agar (Difco). Voges-Proskauer and methyl-red reactions were determined by growing the cultures in V.P.-M.R. medium (Difco), nitrite formation by growing the cultures on nitrate agar (Difco) using the test given for this medium and indol

formation by growing the cultures in tryptophane broth (Difco) and applying the Goré test. Sterile cow serum, 10 per cent, was added to all the above media to assure uniform growth.

The oxygen relationship of the cultures was obtained by growing them in a semi-solid serum liver-infusion agar. Instead of a 2-per-cent agar, as used in the stock medium, a 1-per-cent agar was used. Tubes were filled one-half full with this medium. Two stab inoculations were made in each tube. They were incubated at 37°C. for 10 days, observations being made each day.

AGGLUTINATION TECHNIC

The dry growth of some of the diphtheroids made it rather difficult to obtain uniform suspensions. This was especially true of *C. pseudotuberculosis*. Various methods to overcome this difficulty have been suggested. Bull and Dickinson (1931) described a method whereby they were able to get uniform suspensions of living bacteria by the use of bile salts in the diluting fluid and by shaking and centrifuging. Minett (1922a) was able to get a uniform suspension by grinding the growth, which had been dried over $CaCl_2$, in a mortar and then slowly adding saline solution.

Uniform suspensions of the cultures used in this study were made as follows. Young actively growing cultures were transferred to freshly made serum agar slants. After twenty-four hours' incubation at 37°C. the cultures were washed off with 1 per cent sodium chloride solution and transferred to thick-walled test tubes in which were placed glass beads. The suspensions were shaken for one hour in a mechanical shaker. They were then filtered through compact cotton pads. The cotton pads used in a milk sediment-tester proved satisfactory. The tubes were then centrifuged at low speed for fifteen minutes. The larger clumps were thrown to the bottom of the tubes. The supernatant suspensions were poured into flasks. Saline was added to the residue in the tubes which were again shaken and centrifuged. This procedure was continued until all the bacteria were in a uniform suspension. The suspensions were of the density of tube 2 of McFarland's nephelometer.

Suspensions made in the above manner were used to inoculate rabbits intravenously. None of them were attenuated except *C. diphtheriae*, which was subjected to 60°C. for one hour. The rabbits were given the following doses three days apart, 0.5 cc., 1 cc., 2 cc., 3 cc., 4 cc., 5 cc., for the production of agglutinating serum. The cultures inoculated into rabbits were chosen upon the basis of colony characteristics and fermentation reactions. They were considered to be different species or different strains of the same species. The health of the rabbits was not visibly impaired except in two cases. The rabbits which were given *C. pyogenes*, 8986, and the variant 112a developed subcutaneous abscesses and arthritis.

The agglutination tests were made by the rapid plate method. It was found that a number of the cultures settled during the forty-eight hours required for the macroscopic tube test. The suspensions for the plate test were made by centrifuging the dilute suspensions at high speed until as many of the cells as possible were thrown down. In the dry cultures all were precipitated but in the moist types of growth it was impossible to get all the bacteria out of suspension. The precipitated bacteria were then placed in suspension in 1 per cent sodium chloride solution. The suspensions were standardized to the density of 4 mm. by a Gate's nephelometer and 0.5 per cent phenol was added as a preservative. Equal amounts, 0.04 cc., of suspension and serum were used for preliminary agglutination reactions conducted with the different sera and with each culture. Those which showed a positive reaction were then tested using a standard amount of suspension, 0.04 cc., and varying amounts of serum; 0.08 cc., 0.04 cc., 0.02 cc., 0.01 cc. and 0.005 cc.

The tests were conducted on a glass plate placed on a box in which there was an electric light. The background was black. When agglutination occurred it was present within one minute in all cases. The reactions were very definite.

RESULTS

Morphology

The Corynebacteria are noted for their variability in size and shape. This becomes more apparent when they are grown on the

various types of media. Some of the classical characteristics of these bacteria are their swollen, as well as pointed ends, striations, coccoid shapes, and metachromatic granules. They are nonmotile, and do not produce spores nor capsules. In liquid media they have a decided tendency to form clumps. A parallel (palisade) arrangement of the cells is often observed. This arrangement resembles that of streptococci, but the cells are ovoid, arranged side by side. Some of the Corynebacteria in this study can be recognized by their morphology.

C. pyogenes was the smallest. The morphology of this type is represented in figure 2. It is seen that various shapes ranging from coccoid to bacillary are present. Club-like ends, as well as pointed ends, are visible. On serum agar the cells were large and there was more of a tendency toward the coccoid shape, although a few swollen bacillary forms were observed. The organism growing in milk assumed the bacillary shapes characteristic of those in the direct smear. In serum bouillon *C. pyogenes* was more ovoid than in milk. The formation of groups and the parallel arrangement of the cells was noted. Growth of strain (8986) on Loeffler's serum did not show metachromatic granules when smears were stained with Albert's stain.

C. pseudotuberculosis was distinctly coccoid when grown upon serum agar. Upon careful observation, however, it was seen that the cells were more ovoid. Parallel arrangements were also seen. Granules were formed when the organism was grown on Loeffler's serum medium (fig. 3). The cells were longer on this medium than those on serum agar. The bacteria grown in milk and serum bouillon, were ovoid in shape and tended to form clumps.

C. renalis was the largest of the four species of diphtheroids included in this study. This organism had a distinct bacillary shape in all media. The direct smear (fig. 4) from kidney exudate reveals striated bacilli. When grown on serum agar the cells were generally quite large and uniform, but segmented shapes were seen also. Numerous large metachromatic granules were formed when *C. renalis* was grown on Loeffler's serum medium. Clumping of this organism in serum bouillon and milk occurred.

C. equi showed great variability in shape when grown on various

media. This organism was quite ovoid on serum agar but an occasional bacillary shape was seen (fig. 5). The same coccoid form was found on Loeffler's serum and no granules were formed. When grown in serum bouillon and milk, enormously large cells could be found. When smears were first made from these media, contamination was suggested. Careful plating and staining, however, revealed that swollen bacillary shapes in fluid media were definitely characteristic of this organism.

The morphology of the organisms in Types V and VI did not differ from that of *C. pseudotuberculosis*.

Staining

All of the strains of Corynebacteria used in this study were Gram-positive. However, in the case of *C. pyogenes* a number of Gram-negative cells were found in cultures over three days old. Metachromatic granules were revealed in the majority of the cultures by Albert's stain after the cultures were grown on Loeffler's serum. More granules were seen when the cultures were not over forty-eight hours old. In older cultures the granules which were present were large. Smears from the cultures used in this study were also stained by the Ziehl-Neelsen acid-fast stain after they had grown on modified Petroff's medium and in milk. None of the cultures studied was acid fast.

Macroscopic growth characteristics

The types of diphtheroids described here showed different growth characteristics. *C. pyogenes* grew on serum agar in a very small dew-drop like colony (fig. 6) very similar to the colonies of streptococci. The edge of the colony was entire. Small granules were observed in the center and the outer part of the colony was very finely granular (fig. 7). As the culture aged, the colonies increased in size and became opaque and dry. One strain, 8664, which showed all of the biologic characteristics of *C. pyogenes* was isolated from a case of bovine metritis. This culture grew profusely on all media, was smooth and moist and produced a light yellow-colored pigment.

The growth of *C. pseudotuberculosis* was very dry on all media.

When the colony was small (fig. 8) it was granular and the edge was often uneven. Other colonies the same size showed a central growth from which extended secondary colonies. This gave a pitted, and, in some cases, a mammillated appearance to the colonies when viewed under lower magnification. As the culture aged, it became granular and dry (fig. 9). The entire growth could easily be lifted from the surface of the medium. The color range of the cultures was from a cream to an orange.

C. renalis grew in a moist, cream-colored colony. The edges were irregular, but secondary growth was not observed (fig. 10). The culture became dryer as it aged but never was as dry as *C. pseudotuberculosis*.

The growth of *C. equi* was very distinctive. Small, young colonies were finely granular with smooth edges (fig. 11). The organism grew rapidly, forming a heavy, moist, red-colored streak along the needle track. The growth was distinctly mottled (fig. 12).

Cultures 175 and 176 from milk, placed in Type V, were characterized by adhering so tenaciously to the medium that the growth was removed with difficulty. Otherwise they resembled *C. renalis*. The cultures 181 and 184 in Type VI developed rapidly and were moist and opaque.

In serum bouillon, *C. pyogenes* formed light powdery flakes on the walls and a finely granular sediment in the bottom of the tube. The liquid remained clear. A thick, granular pellicle was formed by *C. pseudotuberculosis* in serum bouillon. As this pellicle aged it fell to the bottom of the tube. In serum bouillon, *C. renalis* produced a white powdery growth which collected along the walls and in the bottom of the tube and in some cultures a thin pellicle was present. The liquid remained clear. Serum bouillon was uniformly clouded by *C. equi*. Very little sediment was formed and no pellicle was produced. Type V, milk cultures, grew in serum bouillon, forming a light powdery sediment. The cultures of Type VI, profusely growing, non-chromogenic organisms, produced a dense turbidity in serum bouillon and formed a thin membranous pellicle.

Biologic reactions

All cultures of the Corynebacteria grew under aerobic conditions upon media to which serum was added. *C. pyogenes*, however, also grew well under anaerobic conditions in a semi-solid serum agar. *C. pyogenes* was the only species which liquefied solidified-bovine serum and gelatin. The reaction of the different types was variable in milk. *C. pyogenes* caused coagulation of milk within forty-eight hours. Digestion of the curd then began and continued slowly until nothing but a very small mass remained in a water-clear whey. During the digestion process the milk had an acid reaction. *C. pseudotuberculosis* grew readily in milk but did not produce any change. The action of the strains of *C. renalis* was variable. Nine of the twenty strains studied caused a digestion of the milk casein with a resulting alkaline reaction but no coagulation. The digestion continued until the medium was of a blue transparent nature. No change occurred in eleven of the cultures. The cultures in Types IV and V did not alter milk. The two cultures of Type VI coagulated the milk and reduced the litmus. There was no digestion of the curd.

C. pyogenes was the only species studied which hemolyzed red blood cells. Indol was not produced by any cultures. None of the types gave a Voges-Proskauer reaction. Only Type VI gave a positive reaction to methyl red. Nitrite was formed by *C. equi* only. Hydrogen sulphide was not present in any of the cultures on lead acetate medium.

Carbohydrate fermentations

C. pyogenes was actively saccharolytic. The fermentation reactions of the strains of this organism studied were quite uniform. One strain, 8664, fermented xylose, salicin and mannitol in addition to the sugars fermented by all the strains, and culture 8986 produced acid in xylose. This reaction was not true of the majority of the strains. *C. pseudotuberculosis* varied in its fermentation reactions. The majority of the strains fermented glucose, levulose, mannose, sucrose and maltose. *C. renalis* also was quite variable in its ability to ferment carbohydrates. Acid

was formed in glucose by the majority of the cultures; many of them fermented levulose and mannose. One strain, 164, was unique in that it fermented only dextrin. *C. equi*, 15655, did not ferment any of the carbohydrates used in this study. Culture 175 Type V caused acid to form in glucose, levulose, galactose and sucrose. The two cultures placed in Type VI were quite active in their attack upon carbohydrates. They produced acid in 11 of the 15 sugars.

TABLE 2

Carbohydrate fermentation

	CULTURE NUMBER	ARABINOSE	XYLOSE	GLUCOSE	LEVULOSE	GALACTOSE	MANNOSE	SUCROSE	LACTOSE	MALTOSE	DEXTRIN	INULIN	SALICIN	DULCITOL	MANNITOL	GLYCEROL
Type I. *C. pyogenes*	8599	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−
	8664	−	±	+	+	+	+	+	+	+	+	−	+	−	+	−
	8986	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Type II. *C. pseudotuberculosis*	63	−	−	+	+	−	+	−	−	+	−	−	−	−	−	−
	163	−	−	+	+	−	−	+	−	+	+	−	−	−	−	−
	4-C-29	−	−	+	+	−	+	+	−	+	−	−	−	−	−	−
	18-O-31	−	−	+	+	+	+	+	−	+	−	−	−	−	−	−
Type III. *C. renalis*	111	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	112	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
	173	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−
	164	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Type IV. *C. equi*	15655	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Type V. Cultures from milk	175	−	−	+	+	+	−	+	−	−	−	−	−	−	−	−
Type VI. Non-chromogenic—profuse growers—moist	181	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+
Type VII. Orange colored variants	112a	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

No gas was produced by any of the cultures. The fermenting action of representative strains is given in table 2.

Agglutination reactions

The sera used in the agglutination reactions were obtained from rabbits inoculated with cultures chosen from the different types. In this study the serum is given the number of the culture.

Serum 34—*Corynebacterium renalis*
Serum 167—*Corynebacterium pseudotuberculosis*
Serum 8664—*Corynebacterium pyogenes*
Serum 8713—*Corynebacterium renalis*
Serum 8728—*Corynebacterium pseudotuberculosis*
Serum 15655—*Corynebacterium equi*
Serum 8772—*Corynebacterium pseudotuberculosis*
Serum 8986—*Corynebacterium pyogenes*
Serum 1310—*Corynebacterium renalis*
Serum 206—*Corynebacterium diphtheriae*
Serum 112a—Orange variant from 112

The strains of *C. pyogenes* were uniform in their agglutination reactions. The five strains studied (table 3) were agglutinated by sera 8664 and 8986. One strain, 9292, from a ewe, was agglutinated by two *C. renalis* sera and one *C. pseudotuberculosis* serum.

C. pseudotuberculosis and *C. renalis* showed a close serological relationship (tables 3 and 4). Four *C. pseudotuberculosis* strains were agglutinated by *C. renalis* serum 34, six by *C. renalis* serum 8713, and four by *C. renalis* serum, 1310. A number of the strains of *C. pseudotuberculosis* failed to be agglutinated by *C. pseudotuberculosis* sera. One serum, 8772, was active in causing agglutination of all of the strains. None of the cultures was agglutinated by *C. pyogenes* serum. Two cultures, 8772 and 809, were slightly agglutinated by *C. equi* serum.

The close relationship between *C. pseudotuberculosis* and *C. renalis* is again emphasized by a study of table 4. Two *C. renalis* strains were agglutinated by *C. pseudotuberculosis* serum 167, eight by serum 8728 and fifteen by serum 8772. More of the strains, however, were agglutinated by sera 34, 8713 and 1310, which were *C. renalis*. Cultures 183, 164, and 165 were placed with the *C. renalis* type because of their fermentation reactions, their morphology, cultural characteristics and because they originated from cases of bovine pyelonephritis. Agglutination reactions, however, indicated a closer relationship to *C. pseudotuberculosis*. Culture 8124, from the semen of a horse, was placed in this type on the basis of its fermentation and agglutination reactions.

C. equi was agglutinated only by its own antiserum.

Since the cultures in Type V could not be made into suitable suspensions, agglutination reactions were not possible.

Type VI cultures were not agglutinated by any of the sera.

Agglutination of Type VII, orange-colored variants, is discussed under heading, "Variation."

It is interesting to note that serum from the rabbit inoculated with culture 296, *C. diphtheriae*, agglutinated two of the *C. renalis*

TABLE 3

Agglutination reactions

	CULTURE NUMBER	SERUM NUMBER										NORMAL SERUM	SALT SOLUTION
		34	167	8664	8713	8728	15655	8772	8986	1310	296	112a		
Type I. *Corynebacterium pyogenes*	8664	−	−	+	−	−	−	−	+	−	−	−	−	−
	8986	−	−	+	−	−	−	−	+	−	−	−	−	−
	8987	−	−	+	−	−	−	−	+	−	−	−	−	−
	4823	−	−	+	−	−	−	−	+	−	−	−	−	−
	9292	+	−	+	+	−	−	+	+	−	−	−	−	−
Type II. *Corynebacterium pseudotuberculosis*	63	+	−	−	+	−	−	+	−	+	−	−	−	−
	163	−	−	−	−	−	−	+	−	−	−	−	−	−
	167	−	+	−	+	+	−	+	−	−	−	−	−	−
	4-C-29	−	−	−	−	−	−	+	−	−	−	−	−	−
	12-C-29	−	−	−	−	−	−	+	−	−	−	−	−	−
	15-C-30	−	−	−	−	−	−	+	−	−	−	−	−	−
	18-O-31	−	−	−	−	−	−	+	−	−	−	−	−	−
	27-C-29	+	±	−	+	±	−	+	−	±	−	−	−	−
	8728	−	+	−	−	+	−	+	−	−	−	−	−	−
	9126	−	+	−	+	+	−	+	−	−	−	−	−	−
	8772	±	−	−	±	±	±	+	−	±	±	±	−	−
	809	±	−	−	±	±	±	+	−	±	±	±	−	−

8599, 8600, 8644 and 8744 were lost before agglutination work was started. + = agglutination in all four dilutions. ± = agglutination in first two dilutions. − = negative.

strains, 34 and 8713, and one strain of *C. pseudotuberculosis*. Sera produced by the injection of these three cultures agglutinated *C. diphtheriae*, 296.

VARIATION

A possibility that variation may occur in diphtheroids was mentioned by Daines and Austin (1931). They wondered

whether the organisms in their group 2 belonged to the same species as group 3. Group 2 was composed of typical Preisz-Nocard bacilli and in group 3 were placed the acid-fast strains which produced deep orange-colored, moist colonies. They ask, "Could these groups be smooth and rough variants of the same

TABLE 4

Agglutination reactions

Type III. *Corynebacterium renalis*

CULTURE NUMBER	SERUM NUMBER											NORMAL SERUM	SALT SOLUTION
	34	167	8664	8713	8728	15655	8772	8986	1310	296	112a		
111	−	−	−	−	+	−	−	−	−	−	+	−	−
174	+	−	−	−	−	−	+	−	+	−	−	−	−
1310	+	−	−	+	−	−	+	−	+	±	−	−	−
9064	+	−	−	+	−	−	+	−	+	−	−	−	−
34	+	+	−	+	+	−	+	−	+	+	+	−	−
112	+	−	−	+	+	−	+	−	+	−	+	−	−
149	+	−	−	−	+	−	+	−	+	−	+	−	−
152	+	−	−	+	−	−	−	−	−	−	−	−	−
182	+	−	−	−	−	−	+	−	−	−	−	−	−
173	+	−	−	+	−	−	+	−	+	−	−	−	−
169	+	−	−	+	−	−	+	−	+	−	−	−	−
74	+	±	−	+	+	−	+	−	+	+	+	−	−
8592	+	−	−	+	−	−	+	−	+	−	−	−	−
8713	+	−	−	+	−	−	+	−	+	−	−	−	−
8714	+	−	−	+	−	−	+	−	+	−	−	−	−
W-2	+	−	−	+	−	−	+	−	+	−	−	−	−
183	−	−	−	−	+	−	−	−	−	−	−	−	−
164	−	−	−	−	±	−	−	−	−	−	−	−	−
165	−	−	−	−	±	−	−	−	−	−	−	−	−
8124	+	−	−	−	−	−	+	−	−	−	−	−	−

+ = agglutination in all four dilutions. ± = agglutination in first two dilutions. − = negative.

species? Or is it possible that there is a symbiosis between these two groups and that they are different species?"

During the course of this study an interesting type of variation was observed. When growth tests were being made of the carbohydrate fermentations an orange-colored colony appeared, with the typical growth of cultures 112, 184 and 27-C-29 from lactose. The carbohydrate had been incubated for 10 days; the

growth tests on Petri dishes had been incubated 4 days and had stood at room temperature 10 days. Variants were also obtained from old milk cultures. These variants were designated 112a, 184a and 27-C-29a. They presented the same morphologic appearance as their parent cultures. The colony characteristics, however, were different. The growth of 112 was semi-dry, rough and cream-colored while 112a was moist, smooth and orange-pigmented. The young colonies under high magnification (× 220) did not exhibit a great deal of difference; both were finely

TABLE 5

Agglutination reactions

	Culture number	Serum number										Normal serum	Salt solution
		34	167	8664	8713	8728	15655	8772	8986	1310	296	112a		
Type IV. *Corynebacterium equi*..	15655	−	−	−	−	−	+	−	−	−	−	−	−	−
Type V. Cultures from milk..	175	The growth of these cultures was of such a tenacious nature that suitable suspensions could not be made for agglutination.												
	176													
Type VI. Non-chromogenic—profuse growers—moist.....	181	−	−	−	−	−	−	−	−	−	−	−	−	−
	184	−	−	−	−	−	−	−	−	−	−	−	−	−
Type VII. Orange-colored variants.....	112a	−	−	−	−	−	−	−	−	−	−	+	−	−
	184a	−	−	−	−	−	−	−	−	−	−	+	−	−
	27-C-29a	−	−	−	−	−	−	+	−	−	−	−	−	−
	95	−	−	−	−	−	−	−	−	−	−	−	−	−
	8774	−	−	−	−	−	−	−	−	−	−	−	−	−
Corynebacterium diphtheriae—Park Strain 8.....	296	+	−	−	+	−	−	+	−	−	+	−	−	−

+ = positive in all four dilutions. − = negative.

granular. Under low magnification (× 10) pit-like depressions could be seen in the growth of 112. The growth appeared uneven. The pit-like depressions were absent in culture 112a and the colonies appeared to coalesce. Strain 184 was a moist-growing culture and was white. Variant 184a was moist but had an orange color. Strain 27-C-29 was a typical rough, dry culture of *C. pseudotuberculosis* while 27-C-29a was smooth, moist and orange in color. The three cultures, 112a, 184a, and 27-C-29a, were devoid of power to ferment the carbohydrates used in this

study. Two strains, 112a and 184a, were agglutinated by serum 112a. It was noted that this serum caused the agglutination of 5 *C. renalis* strains (table 5) and a slight agglutination of 2 *C. pseudotuberculosis* strains (table 5).

Two other cultures, 95 and 8774, were placed in this group of variants. Culture 95 was obtained from the American Type culture collection and was called *C. ovis* (*C. pseudotuberculosis*). Culture 8774, appeared to be similar in all respects to culture 95. Both had a morphology similar to *C. pseudotuberculosis*, but they grew profusely, were moist and smooth and had a deep orange-colored pigment.

No acid-fast staining characteristics were noted in smears made from any of the cultures of Type VII grown on modified Petroff's medium and in milk.

CONCLUSIONS

1. Four distinct species of Corynebacteria are associated with diseases of domestic animals. They are, *C. pyogenes* (Glage) Eberson, *C. pseudotuberculosis* (Preisz) Eberson, *C. renalis* (Enderlen) Ernst and *C. equi* Magnusson.

2. The most distinctive characteristics of *C. pyogenes* are: morphology; aerobic as well as anaerobic growth; reproduction only in media containing blood, blood serum or milk; hemolysin production; coagulation of milk with subsequent digestion of the curd; liquefaction of gelatin and coagulated blood serum; production of acid in, glucose, levulose, galactose, mannose, sucrose, lactose, maltose and dextrin and by some strains, in xylose.

3. *C. pseudotuberculosis* grows in dry granular colonies; does not alter milk, gelatin nor coagulated blood serum; produces acid in glucose, levulose, mannose, sucrose and maltose.

4. *C. renalis* grows in moist isolated colonies; digests milk casein producing an alkaline reaction but has no action on gelatin nor solidified blood serum. It ferments glucose and some strains also ferment levulose and mannose.

5. *C. equi* is characterized by its ability to produce a red-colored pigment, its profuse viscid growth, reduction of nitrates and inability to ferment carbohydrates.

6. *C. pseudotuberculosis* and *C. renalis* are the only two of the four to show a marked serological relationship.

7. Orange-colored variants were isolated from three cultures. These variants resembled their parent cultures in morphology but were smooth and pigmented and failed to ferment carbohydrates whereas the parent cultures were usually rough, cream-colored and demonstrated some fermenting ability.

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PLATE 1

FIG. 1. *C. pyogenes*—Loeffler's serum with potassium tellurite, 48 hours. Natural size.

FIG. 2. *C. pyogenes*—direct smear, lung of calf. Gram stain. × 1400.

FIG. 3. *C. pseudotuberculosis*—Loeffler's serum. Albert's stain. × 1400.

FIG. 4. *C. renalis*—direct smear, kidney exudate. Gram stain. × 1400.

FIG. 5. *C. equi*—serum agar. Gram stain. × 1400.

FIG. 6. *C. pyogenes*, 48 hours, serum agar. × 10.

FIG. 7. *C. pyogenes*, 48 hours, serum agar. × 220.

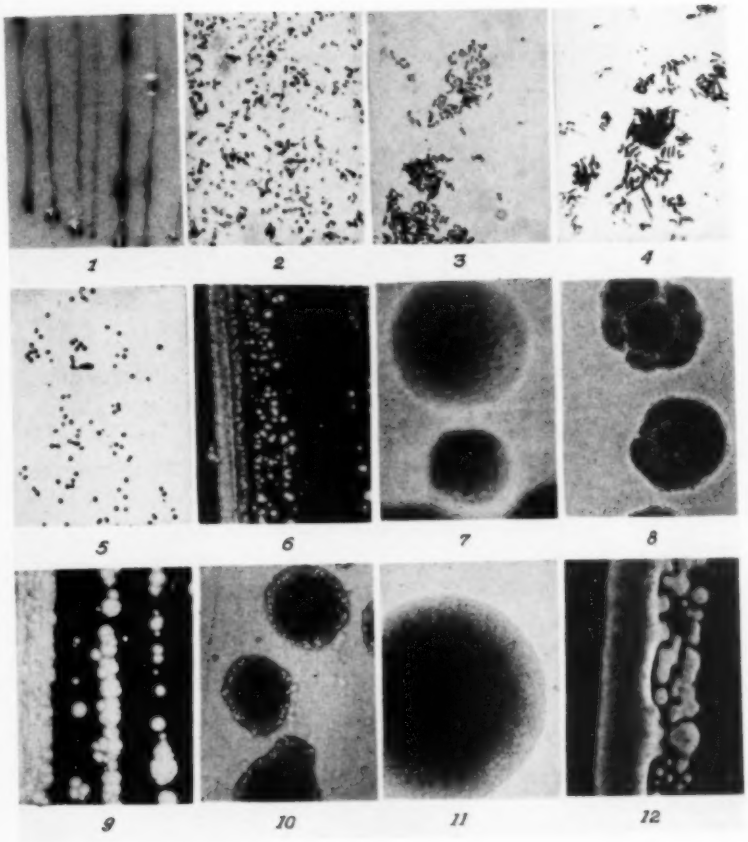
FIG. 8. *C. pseudotuberculosis*, 48 hours, serum agar. × 220.

FIG. 9. *C. pseudotuberculosis*, 4 days, serum agar. × 2½.

FIG. 10. *C. renalis*, 48 hours, serum agar. × 220.

FIG. 11. *C. equi*, 48 hours, serum agar. × 220.

FIG. 12. *C. equi*, 48 hours, serum agar. × 10.



(I. A. Merchant: Study of Corynebacteria)

UNIV. OF MICH.

STUDIES ON PNEUMOCOCCUS VARIATION

II. SMOOTH VIRULENT VARIANTS PRODUCED BY DAUGHTER-COLONY DISSOCIATION OF SMOOTH PNEUMOCOCCUS STRAINS

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INTRODUCTION

A previous paper (Eaton, 1934) described variants of the pneumococcus characterized by a tendency to undergo rapid spontaneous lysis under certain cultural conditions at 37°C. These variants were produced by daughter-colony dissociation of smooth pneumococcus strains on blood agar at 25°C. A further investigation of this method of dissociation revealed that a second and independent type of variation is also produced by the formation of daughter colonies. This consisted in the appearance of a stable smooth variant in the daughter colonies.

The purpose of this paper is to demonstrate that dissociants obtained from the daughter colonies which first appear at 25°C. on the colonies of S pneumococci are smooth strains distinguishable in colony form and other characteristics from the parent smooth strains. The production of these variant S forms has been observed with great regularity in the daughter colony dissociation of most of the pneumococcus cultures of types I, II, III and group IV so far studied. Daughter-colony formation occurs with especial ease in recently isolated strains.

Hadley (1927) has pointed out the frequency of daughter-colony formation in many species of bacteria. He believes that the dissociants formed in daughter colonies are usually not true rough variants. Sometimes these dissociants form large smooth mucoid colonies on subculture, as with organisms of the typhoid

group, and these mucoid colonies may regenerate the parent culture. Other daughter-colony dissociants revert on subculture to the parent colony form, or give rise to rough strains. Daughter-colony variants usually show an increased vigor of growth.

Studies on daughter-colony formation by the pneumococcus on various solid media at 37°C. have usually failed to demonstrate the existence of any distinct dissociants in the daughter colonies except the R forms as reported by Griffith (1928). Often subculture of the papillae has given only smooth mouse-virulent forms apparently similar to the parent type as observed by Paul (1927), Grumbach (1931), and Klumpen (1932). The opaque appearance of the daughter colonies of the pneumococcus was noted by Grumbach. Atkin (1926) reported that the daughter papillae apparently formed no autolysin and were insoluble in bile but recovered their bile solubility and susceptibility to autolysis after being transplanted to fresh medium.

The only essential difference in the method of daughter-colony dissociation described in this paper from that of other workers was that the dissociation was effected and subsequent subcultures were kept at 25°C. instead of at 37°C. This modification has led to the isolation of the phantom colony variants described in the previous paper and the smooth variants previously mentioned and described below.

For the purposes of comparison, the colony form and other characteristics of freshly isolated smooth strains of pneumococcus will be considered normal and these strains will be designated by the letter "N." The variant smooth strains derived from the daughter papillae formed on colonies of the N forms will be designated by the letter "V." In the previous paper these N and V strains were referred to as translucent and opaque colony forms respectively.

METHODS

The media used in the course of these studies were the same as those described in the previous paper, except that 5 per cent rabbit blood instead of horse blood was added to the infusion agar medium used in the latter part of the work. No significant effect

of this change in the source of blood used for the medium was observed.

"Chocolate" agar was made by heating the rabbit-blood infusion agar to 70°C. for thirty minutes with frequent shaking to break up the coagulum.

The N and V strains of pneumococcus were kept in stock culture by cultivation on blood agar at room temperature with transplants every three to four days. Cultivation on blood agar at temperatures around 25°C. appears to result in greater stability of the pneumococcus cultures without affecting their properties in any other way. Once every week or ten days, colonies from the blood agar cultures were transferred to blood broth and grown for twenty-four hours at 37°. Small amounts of these blood-broth cultures were then injected into mice and after the death of the animals the organisms were recovered from the heart blood by cultivation on blood agar at room temperature.

Capsules were stained in smears made by mixing the exudate from the peritoneum of mice dead of pneumococcus infection with a small drop of saline on a slide. The gentian violet and copper sulphate staining method of Hiss, and Wright's stain as ordinarily employed, were the two methods most generally used for demonstrating capsules.

Agglutinin absorptions were done with centrifuged sediments from cultures of pneumococcus in infusion broth containing 0.2 per cent glucose and 5 per cent normal horse serum. Agglutination studies were carried out with organisms centrifuged down from broth cultures and washed, and also with suspensions of organisms from the peritoneum of mice.

PRODUCTION OF S VARIANT STRAINS BY DAUGHTER-COLONY DISSOCIATION

The method of causing daughter-colony formation has been described in the previous paper (Eaton, 1934). Well separated colonies are allowed to age on blood agar at 25° for three to eight days. At some time during this period, usually shortly after autolysis and flattening of the colonies has begun, nuclei consisting of cells of the variant organism appear and these grow above

the surface of the original colony to form the daughter colonies. Subculture of such colonies with daughter papillae to blood agar at 25° gives a mixture of two well defined colony types, the small flat colonies of the original (N) form and the larger more convex colonies of the smooth variant (V). The variant colony forms may be subcultured and passed through mice indefinitely and continue to breed true. They are bile soluble and ferment inulin.

If cultures are allowed to age further at 25°, the first daughter colonies consisting of the smooth variants disappear leaving pits, and later a second growth of daughter colonies appears. These may be shown by subculture to consist of a variety of dissociants most of which are of low virulence or rough.

TABLE 1

The source and type of pneumococcus strains which have been shown to produce smooth variant (V) forms by daughter-colony dissociation

	TYPE I	TYPE II	TYPE III	GROUP IV
Stock cultures	3	1	0	0
Pneumonia sputum	3	1	1	3
Blood cultures	0	1	0	2
Other human sources*	0	0	1	1

* Sources of these strains as follows: type III, mastoid infection; and group IV, pneumococcus meningitis.

When daughter-colony dissociation is allowed to occur at 37° the dissociative processes appear to be more rapid than at 25°. At the higher temperature only rough dissociants have been isolated, in the course of the present studies, from the daughter colonies.

The source and type of the cultures studied in the course of this work are shown in table 1. The seventeen strains tabulated produced smooth V forms by daughter colony dissociation. With three additional stock cultures, one each of types I, II, and III, the formation of smooth V forms could not be demonstrated. It is suspected that these stock cultures may have undergone some dissociative change during long continued artificial cultivation.

None of the freshly isolated strains so far studied has failed to produce smooth variant forms.

Special mention must be made here of the type III culture isolated from a case of mastoid infection. No smooth variants could be obtained from this culture by the ordinary method of daughter colony dissociation. However, when this strain was aged on "chocolate" agar instead of blood agar daughter colonies appeared. By repeated subcultures of the resulting variants and daughter colony formation on blood agar at 25° two distinct V colony forms in addition to the original N colonies were finally isolated. These will be described in the next section.

COLONY CHARACTERISTICS OF THE N AND V FORMS

Colonies of the N and V forms are best differentiated by cultivating the organisms on blood agar at 25° to 28°, but the differences noted in the two forms at room temperature are also quite obvious in colonies grown at 37° under appropriate conditions. Colonies that are well separated exhibit the most striking differences.

The diagrammatic sketches of the colonies in cross section shown in figure 1 represent the comparative changes which take place in the N and V colonies during growth on blood agar at 25°. At the end of twenty-four hours the colonies are small and convex with a smooth shining surface. After two days colonies of the N form have begun to flatten slightly while those of the V form are larger and more convex. At three days a distinct autolysis and flattening has occurred in the colonies of the N form while those of the V form remain convex and continue to grow larger. At four days these changes are still more noticeable. At this time the colonies of most N strains have formed a well defined rim; but occasionally autolysis of the N colonies occurs at the periphery so that pointed colonies are produced. Colonies of the V forms have never been observed to form rims by central autolysis. At four days the surface of the N colonies appears dull and slightly granular while that of the V colonies is mirror-smooth and shining. By transmitted light the N colonies appear translucent

and the V colonies more opaque. By a horizontal light the N colonies appear dull grey and transparent and the V colonies have a greyish white appearance.

Daughter colonies consisting of V dissociants usually appear on colonies of the N form between four and seven days. The V colonies seldom form daughter colonies and these appear only after about ten days. Although autolysis of the N colonies begins on the third day, no autolysis of the V colonies is apparent until after six or seven days.

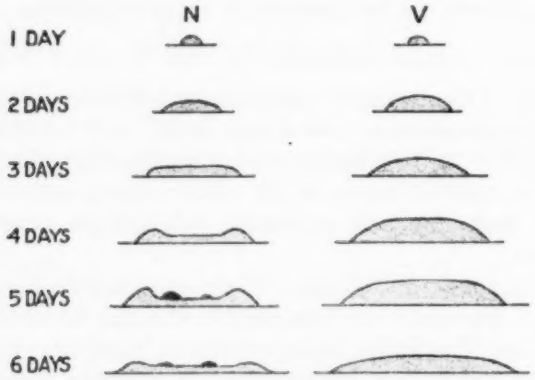

FIG. 1. SKETCHES REPRESENTING APPEARANCE IN CROSS-SECTION OF TYPICAL COLONIES OF N AND V STRAINS AT VARIOUS STAGES OF GROWTH ON RABBIT BLOOD AGAR AT 25°C.

Differences similar to those just described are apparent in some N and V cultures grown at 37° for seventy-two hours, as is pictured in plate 1, figures 9 and 10. With some cultures, however, the differences may be brought out only by growing the colonies in the presence of about one per cent of carbon dioxide. As has been stated in the previous paper the appearance of the V forms in the daughter colony dissociation coincides with the appearance of certain lytic properties in both the N and V strains. These lytic properties are most evident when the organisms are grown at 37° on solid media, and may entirely prevent the appearance of visible colonies or may greatly modify the form of the colonies. How-

ever, the lytic process may be inhibited by small amounts of CO_2 so that the colonies obtained at 37° are similar to those produced at 25°C.

Comparison of the colonies of several type I S stock cultures under identical cultural conditions has revealed that some stock cultures give flat translucent colonies at 25° and 37° which correspond to the N forms, and other stock cultures give convex white colonies corresponding to the V forms.

From one culture of type III as mentioned in the previous section two variant S forms of especial interest were obtained. The first variant, designated V_1 formed flat colonies totally unlike the characteristic large smooth mucoid colonies of the normal strain from which it was derived. These V_1 colonies formed daughter colonies with great ease and the strain corresponded in its other properties to the N forms of other types. From the daughter colonies of the V_1 strain there was isolated a second variant (V_2) which formed moderately large convex, opaque, non-mucoid, colonies and corresponded to the V forms of other types in its general properties. These V_1 and V_2 dissociants of type III bred true, were fully virulent for mice, and agglutinated rapidly and specifically with type III antiserum. They are pictured in plate 1, figures 11 and 12.

ACTION OF THE N AND V FORMS ON BLOOD

Besides the differences in colony shape and surface just described colonies of the N and V forms also exhibit differences in the amount of green coloration produced on chocolate agar and blood agar. On blood agar at 25° to 37° the N colonies produce much wider and deeper zones of greening than do the V colonies. On chocolate agar these differences are somewhat more marked. After four days at 25° the N colonies bleach out the brown color of the medium, but the V colonies cause only a change of the brown color to green. When incubated in whole blood for twenty-four hours at 37°C. the N strains blacken the blood much more rapidly than do most V strains. Both forms grow at about the same rate in whole blood when large inocula are used.

These differences in action on blood by the N and V strains are

presumably due to differences in the metabolic mechanism responsible for hydrogen peroxide formation.

MORPHOLOGY AND CAPSULE FORMATION

By Gram's stain the N and V forms appear identical in morphology, except that pleomorphism and swollen forms are more frequent among organisms of the N strains.

Capsules may be demonstrated by the Hiss capsule stain on both the N and V forms when stained in smears directly from the peritoneum of mice, or after growth in serum or whole blood. In general the capsules of the V forms stain more lightly, by the method of Hiss, and appear thinner than those of the N forms. The capsules of the N forms sometimes appear to contain dark-staining granules. These differences are illustrated in plate 1, figures 1 and 2.

By using Wright's stain it has also been possible to demonstrate differences in capsule-staining reactions not only among some of the N and V forms but also between the pneumococcus types I, II, and III. Seastone (1934) has recently used Wright's stain to demonstrate capsules in young cultures of streptococci. By this staining method the following differences in capsule formation in smears from the mouse peritoneum have been noted in the pneumococcus.

Type I-N capsules appear as red-staining ragged fringes around the organism. Type I-V, capsules do not stain by the method of Wright (plate 1, figures 3 and 4). These differences have been observed in the N and V forms derived from four strains of type I.

Type II-N and V capsules do not stain by Wright's method although they are readily demonstrated by the Hiss Capsule stain (plate 1, figures 5 and 6).

Type III-N, -V_1, and -V_2 capsules all stain by the method of Wright as broad pink fringes about the organisms. The capsules of the N and V_1 forms appear similar in staining reaction, but the V_2 capsules appear slightly narrower and more striated than those of the N and V_1 forms. Differences between the V_1 and V_2 capsules are illustrated in plate 1, figures 7 and 8.

DISSOCIATIVE BEHAVIOR OF THE N AND V FORMS

Significant differences have been observed between the N and V forms as regards dissociative behavior. The N forms readily produce daughter colonies on blood agar at 25° and also at 37°, thus giving rise to many dissociant forms including the smooth V, intermediate forms, and R forms. The V forms produce daughter colonies slowly or not at all at 25° or 37° and dissociants differing appreciably from the parent V strains can seldom be isolated from the daughter colonies. Certain strains which appear to be intermediate between N and V forms in their properties may give rise to more typical V strains by daughter-colony dissociation after a period of ten to twelve days at 25°.

Unless cultivated with frequent transplants, mouse passage, and colony selection the N forms cannot be kept in pure culture unmixed with V or R dissociants. When N forms are transplanted repeatedly at 25° or 37° without mouse passage they dissociate abruptly after several subcultures to the R variant. If allowed to remain without transplanting for any length of time at temperatures between 25° and 37° on agar or in broth the N forms may dissociate to smooth V forms.

The V strains are much more stable than the N forms. They do not readily dissociate to the R variant even after prolonged cultivation without mouse passage. When grown under cultural conditions which readily produce R dissociants from the N strains, the V strains show no change in colony form and only a gradual diminution in virulence for mice. After cultivation for several months under such unfavorable conditions V strains may eventually become rough by a transition through intermediate forms but abrupt dissociation to the R variant does not occur.

These statements are based on observations of about twenty N and V strains of pneumococcus which were carried as stock cultures under various conditions and for various lengths of time over a period of three years.

TYPE SPECIFICITY AND VIRULENCE FOR MICE

The variant smooth strains which have just been described agglutinated specifically with the same type of pneumococcus

antiserum as did the N strains from which they were derived. No cross-agglutinations with sera of other types have been observed with fully virulent smooth V strains. Both the N and V strains of types I and II killed mice in forty-eight hours at dilutions of 10^{-7} to 10^{-8}. The N, V_1, and V_2 strains of type III killed mice in forty-eight hours at dilutions of 10^{-6} to 10^{-7}.

As was indicated in the previous section "degraded" V forms may be produced by prolonged cultivation of the smooth V strains with infrequent transplants. Under these conditions the colony form remains smooth and convex and the organisms grow diffusely in 10 per cent normal horse serum broth but the virulence for mice gradually decreases, and eventually there is a partial loss of type specificity as indicated by agglutination tests. These degraded V forms apparently correspond in a general way with the forms intermediate between S and R, described by Klumpen (1932), Blake and Trask (1933) and Paul (1934). The virulence and type specificity of many of these "degraded" strains may be restored by repeated mouse passage.

AGGLUTININ ABSORPTION AND MOUSE PROTECTION TESTS WITH ANTI-SERA FOR THE N AND V STRAINS

Anti-sera were prepared in rabbits by intravenous injection of heat-killed cultures of the N and V forms of types I and II, and also by injection of formalinized cultures of N and V forms of type I in two animals.

In table 2 are presented the results of agglutination tests with absorbed and unabsorbed anti-sera against the N and V forms of type I and II. The N anti-sera were cross-absorbed with V organisms of the same type, and the V anti-sera were absorbed with N organisms. The absorptions were repeated until no agglutination of the absorbing organisms occurred. From the results tabulated it may be seen that V organisms absorb the agglutinins for the N and *vice versa*. The antigenic compositions of the V and N strains, by these tests, therefore appear to be identical.

Tests for cross protection by type I-V and -N anti-sera in mice were done by injecting simultaneously 0.1 cc. of the N or V anti-sera and various amounts of twenty-four-hour blood broth

cultures of the N or V strains. The results may be summarized as follows.

1. Mice protected with 0.1 cc. of N antiserum: all lived with 0.02 cc. or less of either N or V cultures; and all died with 0.05 cc. or more of either N or V cultures.[1]

2. Mice protected with 0.1 cc. of V antiserum: all lived with 0.15 cc. or less of either N or V cultures; and all died with 0.5 cc. or more of either N or V cultures.[1]

Although the I-V antiserum had a protective value about ten times as great as the I-N antiserum, it may be seen from these results that either serum produced the same degree of protection against the I-N as it did against the I-V strain.

TABLE 2

Agglutinin absorption tests with N and V strains of types I and II

ANTISERUM	ABSORBED WITH	STRAIN AGGLUTINATED	AGGLUTININ TITRES OF SERA	
			Before absorption	After absorption
I-N	I-V	I-N	1:120	<1: 7
I-V	I-N	I-V	1: 50	<1: 7
II-N	II-V	II-N	1:200	<1:10
II-N	II-V	II-V	1: 60	<1:10
II-N	II-V	II-N	1: 50	<1: 5
II-V	II-N	II-V	1: 20	<1: 5

ACTIVE IMMUNIZATION OF MICE AGAINST N AND V STRAINS OF TYPE I

Mice were immunized with blood-broth cultures of the N and V strains of type I killed with merthiolate. Each mouse received into the peritoneum one dose of 0.3 cc. and two doses of 0.5 cc. over a period of twelve days. Sixteen mice were immunized with each strain. Of the group of mice receiving the N vaccine five became emaciated and died (with sterile hearts' blood) during the course of immunization. None of the mice receiving the V vaccine died.

[1] Each 0.1 cc. of cultures contained approximately 5 million minimal lethal doses of I-N or I-V.

The mice were tested sixteen days after the last immunizing injection. Test doses of 0.1 and 0.01 cc. of twenty-four-hour blood-broth cultures of the N and V strains were injected intraperitoneally into mice immunized with each strain. The results of these tests are presented in table 3. Only one of the mice receiving 0.1 cc. of the test cultures survived while over half of those receiving 0.01 cc. survived. Therefore, the fatal dose of either N or V cultures for mice immunized with either N or V strains is between 0.1 and 0.01 cc. There seems to be little difference in the immunity conferred by either strain.

TABLE 3

Active immunization of mice with type I-N and I-V strains

IMMUNIZING STRAIN	TEST STRAIN	AMOUNT OF TEST STRAIN INJECTED*	NUMBER OF MICE TESTED	NUMBER OF DEATHS
		cc.		
I-N	I-N	0.1	3	2
I-N	I-N	0.01	3	1
I-N	I-V	0.1	3	3
I-N	I-V	0.01	2	0
I-V	I-N	0.1	3	3
I-V	I-N	0.01	4	2
I-V	I-V	0.1	3	3
I-V	I-V	0.01	4	1

* 0.1 cc. of a twenty-four-hour blood broth culture contains approximately 5,000,000 minimal lethal doses.

DISCUSSION

The daughter-colony dissociation of the pneumococcus on blood agar at 25°C. resembles the dissociation of other bacterial species on solid mediums. It is interesting to note that the N cultures which are very susceptible to autolysis on solid mediums form daughter colonies and dissociate much more readily than do the V dissociants which are relatively resistant to autolysis and show much less tendency to dissociation. Although no bacteriophage for the pneumococcus has as yet been demonstrated, this dissociative behavior is analogous to that of other species of bacteria in the presence or absence of bacteriophage. In the pneumococcus, stability toward dissociation and increased

vigor of growth on solid media are also accompanied by a decreased activity of the metabolic mechanism which oxidizes hemoglobin to methemoglobin and its decomposition products. It is possible that further studies along these lines may help to elucidate the mechanism of bacterial dissociation.

As regards virulence for mice and antigenic composition as studied in the course of this work the daughter-colony V dissociants are indistinguishable from their smooth parent strains. For this reason they cannot be classed as forms intermediate between smooth and rough. It is possible that differences in antigenic composition between the N and V strains may be detected by some means other than those reported in this paper. Perhaps such antigenic differences are indicated by the staining reactions of the capsules. On the basis of present evidence it seems probable that the pneumococcus variations reported in this and the previous paper are changes independent of the smooth to rough dissociation.

Differences in the colony form of various smooth strains of pneumococcus of the same type have been noted by many investigators. The present paper represents, to our knowledge, the first description of the production of stable smooth variants under controlled experimental conditions. In view of the observed marked instability of freshly isolated N strains of pneumococcus on blood agar at 25° or 37°C. it seems reasonable to postulate that many freshly isolated cultures of pneumococcus dissociate to forms resembling the smooth V strains during prolonged cultivation on artificial mediums even if virulence is maintained by mouse passage. We have definitely observed at least one such change from the N type of colony to the V form in a stock culture maintained by daily transplants in blood broth with mouse passage once a week. Many of the stock cultures examined during the course of this work formed colonies intermediate between the N and the V type in appearance, and practically all of the stock cultures studied produced daughter-colony dissociants less easily than freshly isolated strains, thus resembling the V forms. A few stock strains showed decreased methemoglobin formation on blood agar, formed daughter colonies with

great difficulty or not at all, and resembled typical smooth V dissociants in colony form.

SUMMARY

Some stock strains and all of the freshly isolated strains of smooth pneumococcus studied during the course of this work gave rise by daughter-colony dissociation on blood agar at 25°C. to smooth variants which differ in colony form from the parent strains.

The smooth variant (V) forms and the smooth parent (N) strains from which they were derived have the same virulence for mice and do not differ in antigenic composition as determined by agglutination, agglutinin absorption, and mouse protection studies.

The smooth V strains are very stable. They form daughter colonies and dissociate to R forms much less easily than do the N or freshly isolated strains.

The N and V strains appear also to differ in capsular staining reactions, and in the ability to form methemoglobin in blood.

The significance of these observations with regard to bacterial dissociation, and the maintenance of smooth pneumococci in stock cultures is discussed.

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PLATE

PLATE 1

In figures 1 to 8 the pairs of stained preparations shown in each horizontal row were stained on the same microscope slide so that in comparing the capsule stains of the N and V forms the effects of variations in the method of staining were minimized. All stained preparations were made from the mouse peritoneum.

FIG. 1. Type I-N strain from mouse peritoneum stained by Hiss capsule stain. Capsules appear dark and granular.

FIG. 2. Type I-V strain stained by Hiss method. Capsules colored a light pink.

FIG. 3. Type I-N strain by Wright's stain. Capsules appear as ragged red stained fringes.

FIG. 4. Type I-V strain by Wright's stain. Capsules are not stained.

FIG. 5. Type II-N strain stained by Hiss capsule stain. Capsules darkly colored.

FIG. 6. Type II-V strain by Hiss stain. Capsules are stained very lightly on some of the organisms, not at all on others.

FIG. 7. Type III-V_1 strain stained by method of Wright. Capsules appear as wide pink staining halo.

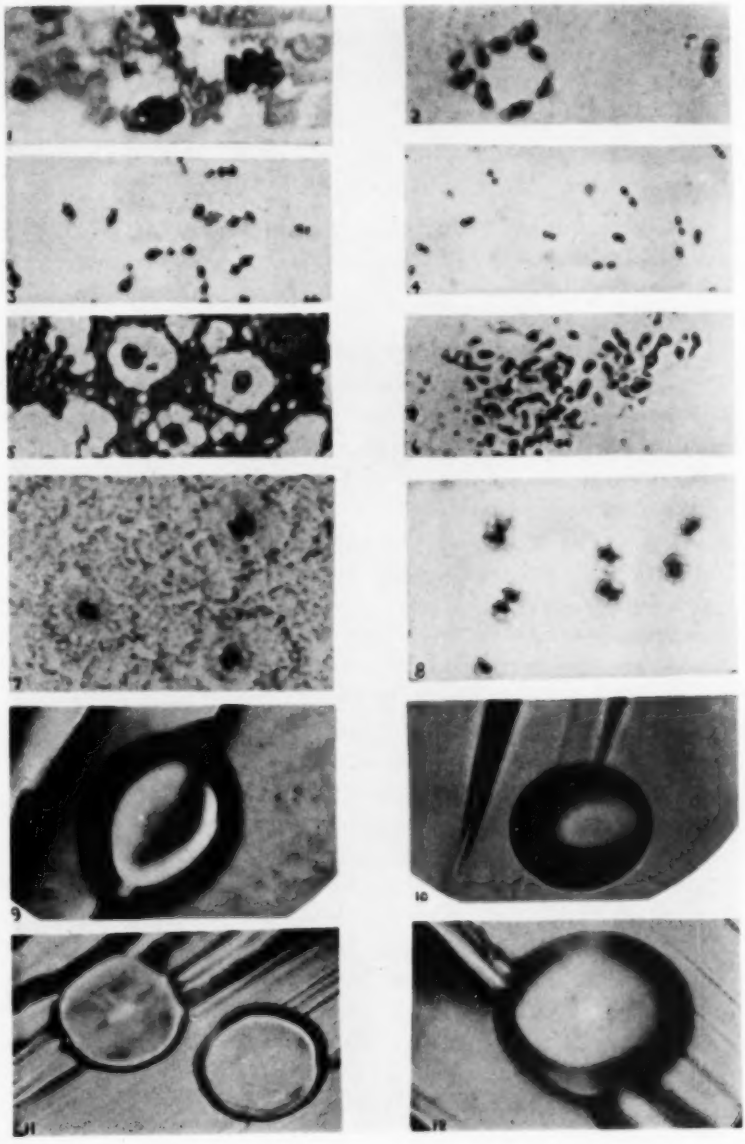
FIG. 8. Type III-V_2 strain by Wright's method. Capsules are narrower than those of V_1 and appear striated.

FIG. 9. Colony of type I-N strain after growth of forty-eight hours on blood agar at 37°C.

FIG. 10. Colony of type I-V strain on other half of same agar plate as colony in figure 9.

FIG. 11. Colonies of type III-V_1 strain after growth of four days at 25°C. on blood agar.

FIG. 12. Colony of type III-V_2 strain on other half of same agar plate as colony in figure 11.



(Monroe D. Eaton: Studies on Pneumococcus Variation)

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STIMULATION OF BACTERIAL GROWTH RATE BY METHYL GERMANIC OXIDE

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Methyl germanic oxide has been found to exert a stimulating action on arbacia eggs (unpublished experiments by Dr. I. R. Taylor), and it seemed interesting to determine its effect on bacteria. The effect of 0.01 M methyl germanic oxide in nutrient broth was studied. This concentration represents the approximate limit of solubility of the compound, and lower concentrations give less pronounced effects.

A preliminary macroscopic test was made, using 100 strains of nine genera of cocci and colon-typhoid bacteria. Turbidity observations were sufficient to show that all the colon-typhoid strains and approximately 40 per cent of the strains of cocci were stimulated at some time during their growth period. The extent of this stimulation was then studied by constructing growth curves from plate counts at suitable intervals. The temperature of all experiments was 37°C. Inoculations were made with one drop of sixteen-hour broth cultures. In order to eliminate slight chance variations in the extent of growth, triplicate tubes were pooled and dilutions made from this mixture. The reproducibility of experiments is shown by the fact that the average probable deviation of all experiments reported in this paper was 7.5 per cent.

Figure 1 is an average of eight experiments on one strain of *Escherichia coli*. The curves show clearly that the effect attributed to the methyl germanic oxide depends on the time at which observations are made. Thus, while a pronounced stimulation was apparent at the third hour in germanium broth, and the maximum number was reached in eight hours, the decline in numbers which began immediately thereafter resulted in a

crossing of the growth curves at about twenty-one hours. Beyond this cross-over point the viable count in the germanium broth drops below the viable count of the control. If the only counts made in the experiment had been on cultures older than twenty-one hours, the germanium compound would have appeared to exert a mildly toxic effect.

However, if the ratio of the area under the experimental curve to the area under the control curve for a definite period is used as a measure of stimulation, a different conclusion will be reached.

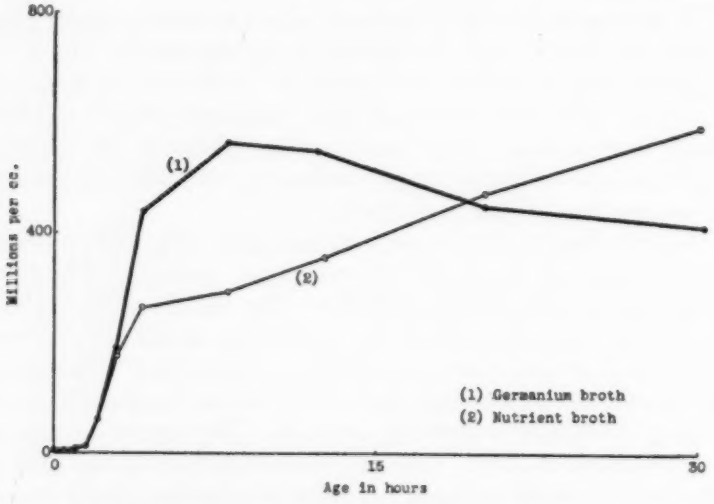

FIG. 1. STIMULATION OF ESCH. COLI BY METHYL GERMANIC OXIDE

The area under a growth curve expresses the total growth in *cell-hours*, since it is a function of the number of cells produced and the time during which they are in existence. As noted previously, *Esch. coli* in germanium broth reaches its peak of growth at eight hours. Hence, the ratio of the areas up to this point is an index of the total stimulating action during the period of growth of the culture in germanium broth. This *ratio of stimulation* for *Esch. coli* is 1.65. That is, cultures in methyl germanic oxide broth showed 65 per cent more total cell-hours of growth than did control cultures.

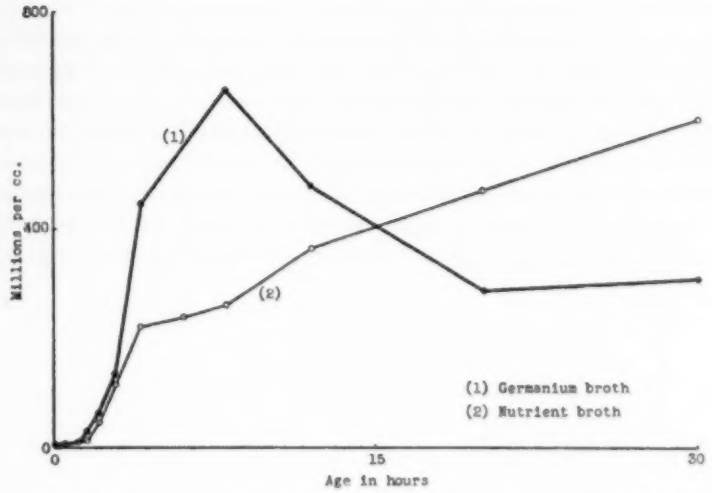

FIG. 2. STIMULATION OF A. AEROGENES BY METHYL GERMANIC OXIDE

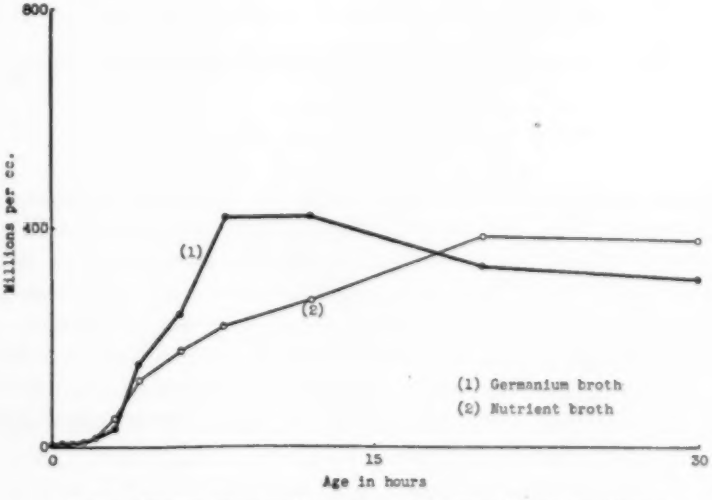

FIG. 3. STIMULATION OF EB. TYPHI BY METHYL GERMANIC OXIDE

Experiments with other species gave similar results. The average of eight experiments with a strain of *Aerobacter aerogenes* is shown in figure 2. Stimulation was more pronounced than with *Esch. coli*, beginning one hour after inoculation. The drop from the peak of growth at eight hours was likewise steeper. The cross-over point in this case occurred at fifteen hours. The ratio of stimulation in cell-hours from the beginning of the experiment to the point of maximum numbers in germanium broth was 2.08. In ten experiments with a strain of *Eberthella typhosa* (fig. 3),

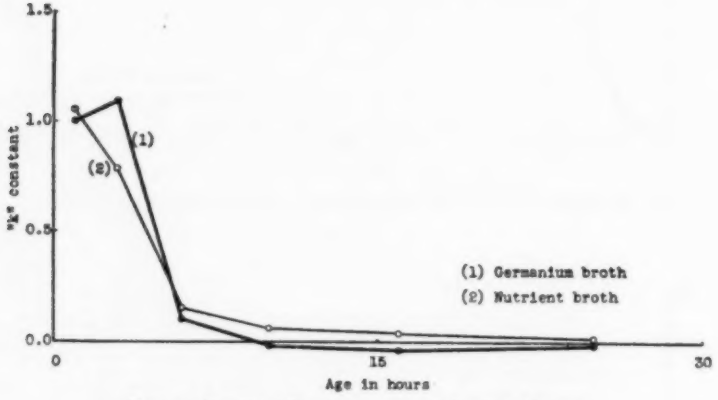

FIG. 4. AVERAGE STIMULATION OF GROWTH RATES

stimulation began at four hours, with the maximum numbers in germanium broth at eight hours, and the cross-over point at eighteen hours. In this case, the ratio of stimulation in cell-hours was 1.42. The effects on *Bacillus subtilis*, *Micrococcus cereus*, and *Staphylococcus albus* were not essentially different.

Further light on the stimulation process is gained from a study of growth rates. The curves in figure 4 represent an average of all experiments performed. Growth rates were calculated according to Slator's formula:

$$k = \frac{\ln b - \ln a}{t}$$

where a is the number of bacteria at the beginning of time t, and b is the number at the end of time t. Constants are plotted on the graphs at the mid-points of the time intervals represented. During the first two hours, the period preceding the logarithmic growth phase, germanium broth cultures show a lower growth

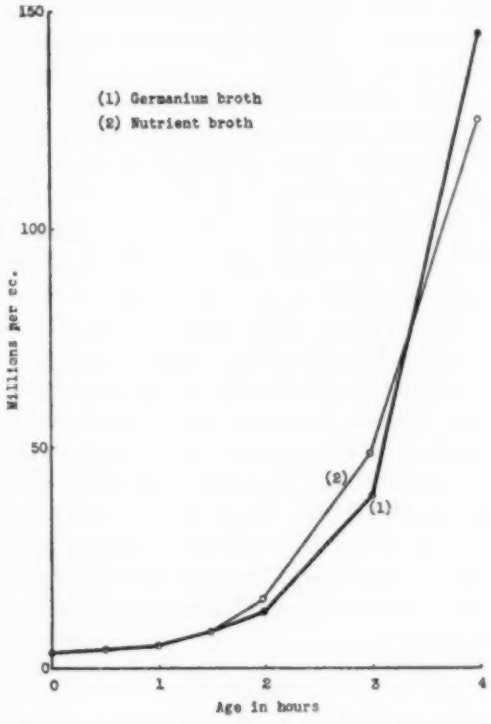

FIG. 5. INHIBITION OF EB. TYPHI DURING LAG PHASE

rate than controls. During the next two hours, the control growth rate falls, while the experimental growth rate rises to a peak. Stimulation of the growth rate amounting to 37.2 per cent is thus produced during the early logarithmic phase. The growth rate drops during the succeeding interval to a point below that of the control.

The difference in the value of k during the first two hours suggests a prolongation of the lag phase. That this is actually the case is indicated by experiments in which counts were made at half-hour intervals. Figure 5 is an example of these.

It is probable that many substances which affect the growth of bacteria act similarly to methyl germanic oxide. In most cases it has not been clearly recognized that a given concentration of a substance may have a stimulating action at one phase of the growth cycle and an inhibitory or toxic action at other phases. Methyl germanic oxide acts in at least two ways. It is inhibitory during the lag phase, which is prolonged. It stimulates the rate of growth so that the point of maximum numbers is reached much earlier than in controls. It may have a toxic action on cultures beyond the maximum stationary phase of growth. However, this is by no means certain. The decline in numbers may be simply premature, not hastened by the germanium compound but occurring earlier than in the controls in consequence of the shorter period of increasing numbers.

Many of the salt effects reported in the literature were determined by single counts, frequently on twenty-four-hour cultures. From the growth curves presented here it is evident that a twenty-four-hour count would have shown 0.01 M methyl germanic oxide to be a toxic concentration. If the entire curves are taken into account, however, there is no direct evidence of toxic action. Rather, there is stimulation, ranging from 40 to 100 per cent during the short period in which the experimental and control cultures are both growing.

CONCLUSIONS

There are three differences between cultures in methyl germanic oxide broth and in nutrient broth. The cultures in methyl germanic oxide (1) are inhibited during the lag phase, (2) are stimulated during the early logarithmic phase, and (3) show a premature period of decline.

Acknowledgment is due to Dr. E. A. Flood of the Department of Chemistry, who supplied the methyl germanic oxide used in these experiments.

CAN AZOTOBACTER CHROOCOCCUM SYNTHESIZE VITAMIN D?[1]

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Azotobacter chroococcum, together with other nitrogen-fixing microörganisms, possess even greater synthetic powers than do higher plants, in that it can build complex organic compounds, including proteins, from elementary nitrogen and inorganic salts, provided a suitable source of energy is available. Hence, it seems reasonable to expect that this organism may be able to synthesize vitamin D or its precursor from such a simple substrate.

In order to test this thesis, a strain of *Azotobacter chroococcum* was cultured at 26° to 28°C. in media of the following composition:

Monopotassium phosphate	0.02	per cent
Magnesium sulfate	0.02	per cent
Sodium chloride	0.02	per cent
Calcium sulfate	0.01	per cent
Iron as ferrous sulfate	50	p.p.m.
Iodine as sodium iodide	40	p.p.m.
Manganese as manganous carbonate	40	p.p.m.
Calcium carbonate	1.0	per cent
Mannitol	1.5	per cent
Distilled water	1000.0	cc.

All chemicals were analyzed and of the highest purity. At the end of three weeks' incubation, each flask had a profuse growth

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[2] Chemist and bacteriologist.

[3] The author wishes to express his appreciation to the following who have assisted in the work: Dr. Ethelyn O. Greaves, Wendell Reeder, and Harold Nielsen.

and yielded approximately one gram of dry cells. To insure purity, the cultures were periodically examined.

After considerable experimentation, the following method was used for separating the cells from the culture medium: Dilute hydrochloric acid was added to each flask to bring the calcium carbonate into solution. The bacterial suspension was then filtered by suction through Buchner filters. The cells were washed with small quantities of distilled water. While still moist, the growth was removed from the filter, dried for eight hours at 70°C., and then pulverized.

TABLE 1

Average daily gain and average daily consumption of food by rats on various diets

DIET	FOOD EATEN DAILY	DAILY GAIN
	grams	*grams*
Steenbock's	7.8	1.0
*Steenbock's + 0.75 unit vitamin D daily from beginning	8.2	1.0
Steenbock's + 0.5 gram Azotobacter cells daily	7.4	1.2
Steenbock's + 0.5 gram irradiated Azotobacter cells daily	6.9	1.2
Steenbock's + 0.75 unit vitamin D daily after rickets developed	8.5	1.2
Steenbock's + 3.0 units vitamin D daily	9.0	1.1
Steenbock's + 25.0 units vitamin D daily	6.9	1.0
Steenbock's + 75.0 units vitamin D daily	8.5	1.0

* Calculated according to Steenbock's units.

The growth was found to contain 18 per cent total nitrogen and the essential amino acids—arginine, histidine, cystine, and lysine (Greaves and Reeder, 1935). The bacterial cells were assayed for vitamin D as follows: Rats were bred and reared on Steenbock's stock diet. When twenty-four days old the young were weaned (their weight being from 50 to 60 grams), placed in metal cages (bedded with sterilized shavings), and kept in a dark room. They were fed Steenbock's rachitogenic diet number 2965 (Hawk and Bergeim, 1931) until they developed rickets as shown by appearance, the line test, and phosphorus content of the blood of sacrificed animals. This required twenty-four days. The rachitic animals were kept for another five days on Steenbock's

rachitogenic diet. In some cases this diet was supplemented with Squibb's viosterol, in others with *Azotobacter chroococcum* cells, and in still others with irradiated *Azotobacter chroococcum* cells. Weighed quantities of the Azotobacter cells were fed to each animal separately. A record was kept of the food consumed and the daily weight of each animal. The average results for food consumption and gain in weight are given in table 1.

No animal ate less than 6.9 grams per day and all made satisfactory gains in weight. On the fifth day the animals were bled and the blood analyzed for phosphorus by the Youngberg method

TABLE 2

Results of line tests on animals receiving rachitogenic diets alone and supplemented with Squibb's viosterol, or Azotobacter chroococcum cells

DAILY DIET	LINE TEST
Positive control:	
Steenbock's + 0.75 unit Vitamin D from beginning	Normal
Negative control:	
Steenbock's	Severe rickets
Steenbock's + 0.5 gram daily, Azotobacter cells	Severe rickets
Steenbock's + 0.5 gram irradiated Azotobacter cells	++
Steenbock's + 0.75 unit vitamin D after rickets developed	++
Steenbock's + 3.0 unit vitamin D	+++
Steenbock's + 25.0 unit vitamin D	+++
Steenbock's + 75.0 unit vitamin D	++++
Control, Steenbock's diet, killed at end of 3 weeks	Severe rickets

(Hawk and Bergeim, 1931). The right tibia was used for the line test; the other was analyzed for calcium and phosphorus. The line test was made according to McCollum and coworkers (Hawk and Bergeim, 1931); the phosphorus was determined according to the Youngberg method (Hawk and Bergeim, 1931), and the calcium according to the Kramer-Howland method (Kramer and Howland, 1926). Average results for the line test are given in table 2.

The 0.5 gram Azotobacter cells would add to the diet 2.8 mgm. of phosphorus, but this was not the determining factor in healing as no healing occurred in those animals fed the unirradiated cells.

Positive controls had normal tibia, whereas all animals which

had been on Steenbock's rachitogenic diet for both the twenty-one days and twenty-six days had severe rickets. This was also true with the animals receiving Steenbock's rachitogenic diet supplemented with 0.5 gram daily of Azotobacter cells. However, in the case of all animals receiving irradiated *Azotobacter chroococcum* cells, there was unmistakable evidence of healing, thus indicating that *Azotobacter chroococcum* had synthesized ergosterol which had been transformed by irradiation into vitamin D. The quantity synthesized was 1.5 Steenbock's unit per gram of *Azotobacter chroococcum* cells. If *Azotobacter chroococcum* produced vitamin D, it was not in sufficient quantities to cause healing in the time and with the quantity fed.

TABLE 3

Average milligrams of phosphorus in 100 cc. blood, as well as percentage of ash, phosphorus, and calcium in tibia of rats on various diets

DAILY DIET	P (MGM. PER 100 CC. BLOOD)	COMPOSITION OF BONE		
		Ash	P	Ca
		per cent	*per cent*	*per cent*
Positive control:				
Steenbock's + 0.75 unit vitamin D from beginning	3.60	26.40	6.12	13.77
Negative control:				
Steenbock's	2.70	23.13	5.10	11.42
Steenbock's + 0.5 gram Azotobacter cells	2.74	24.35	5.17	10.24
Steenbock's + 0.5 gram irradiated Azotobacter cells	3.14	25.20	5.76	10.69
Steenbock's + 0.75 unit vitamin D after rickets developed	3.36	23.02	5.03	8.68
Steenbock's + 3.0 unit vitamin D	4.11	25.16	5.07	10.08
Steenbock's + 25.0 unit vitamin D	3.49	23.26	5.45	9.58
Steenbock's + 75.0 unit vitamin D	3.81	25.75	5.71	12.89
Control, Steenbock's diet, killed at end of 3 weeks	2.67	24.32	5.49	10.33

The blood of the animals was analyzed for phosphorus, and although a five-day period is rather limited to expect definite chemical changes to occur in the bones, yet they were analyzed for ash, calcium, and phosphorus. Average results are given in table 3.

It is evident from the blood picture that the animals were on the rachitogenic diet long enough to become rachitic and that the feeding of the nonirradiated *Azotobacter chroococcum* cell had no influence, whereas the feeding of the irradiated Azotobacter cells increased the amount of phosphorus in the blood to that found in the blood of normal animals.

It is apparent that the animals were not on the curative diets long enough to show striking changes in the composition of the bone, yet the results bear out the conclusion obtained with the line test and blood analysis that *Azotobacter chroococcum* can synthesize the precursor of vitamin D and that if vitamin D itself was produced, it was in too small quantities to manifest itself in the short time fed at such a low level.

SUMMARY

Azotobacter chroococcum was cultured on synthetic media consisting of inorganic salts and mannitol and tested for vitamin D and its precursor by feeding to rats. It was concluded from the line test, the phosphorus content of the blood, and the composition of the bone ash that *Azotobacter chroococcum* synthesizes from this simple medium the precursor of vitamin D, which is readily transformed into vitamin D by irradiation. This finding places this microörganism along with *Clostridium butyricum* (Heiduschka and Lindmen, 1929), *Staphylococcus albus* (Gerard, 1895), and the higher plants (Bills, 1935) in being able to synthesize ergosterol but unable to synthesize vitamin D. It differentiates *Azotobacter chroococcum* from *Mycobacterium tuberculosis* (Prickett and Massengale, 1931), *Bacterium coli*, and Corynebacterium (Behring, 1930) in that they contain neither sterole nor vitamin D.

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THE INTERRELATION OF THE AMOUNT OF V-FACTOR AND THE AMOUNT OF AIR NECESSARY FOR GROWTH OF HEMOPHILUS INFLUENZAE TYPE b IN CERTAIN MEDIA

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During a study of *Hemophilus influenzae* in which these organisms were grown in immune serum, in fresh normal serum, and also in a medium containing no serum, it was observed that when certain of the cultures were kept at rest during incubation no growth resulted, whereas when similar cultures were kept in motion growth took place. An investigation was therefore undertaken to study the nature of this phenomenon and to determine, if possible, its dependence upon the action of accessory growth factors.

A number of studies on the accessory substances required for the growth of *H. influenzae* have been reported. (See reviews by Scott (1929), Anderson (1931), and Thomson and Thomson (1933).) It has been shown that red blood cells contain two substances which are essential for the promotion of the growth of these bacteria, and that growth can occur in the absence of blood derivatives if plant tissue is present. The accessory factors were designated X and V by Thjötta and Avery (1921), who made a very intensive analysis of these substances. These authors found that the X-factor is always associated with that fraction of the blood which gives a peroxidase reaction with benzidine, and suggested that it acts as a catalyst. Olsen (1920) had previously stated that the guaiac and benzidine reactions go hand in hand with the ability of blood derivatives to support growth of *H. influenzae*. This author suggested that hemoglobin acts as a catalytic agent in rendering oxygen available for the bacilli.

Fildes (1921) also presented the view that iron-containing pigments function as catalysts in accelerating the transfer of oxygen to the bacilli.

More recently, it has been reported that under anaerobic conditions the X-factor is not essential for growth. (Kopp (1927–8), Eirund (1929), and Anderson (1931).) Kopp has suggested that the X-factor has the property of protecting the influenza bacillus from the action of oxygen which is harmful to it. Anderson thinks that it acts "in rendering harmless an otherwise harmful substance formed in the presence of atmospheric oxygen."

Thjötta and Avery suggested that the action of the V-substance is similar to that of vitamines in animal nutrition. Subsequently, Avery and Morgan (1924) suggested that the action of both the X and V-factors may be related to the physiological processes of cell respiration; and, in a comparison of the growth-promoting factors of plant tissue with the X and V-factors of blood, they state that the peroxidase and catalase of plant tissue are related, in function at least, to the catalytic properties of the X-factor in blood, while the V-substance may represent a co-ferment adapted to a particular oxidase system.

In the present investigation influenza bacilli have been cultivated under conditions in which the supply of air is varied, (1) in Levinthal broth, (2) in Levinthal broth containing an additional amount of the V-factor, (3) in the presence of fresh rabbit serum to which had been added a small amount of the V-substance, and (4) in the same serum to which had been added a larger amount of the V-substance. An adequate supply of the X-factor was present in all of the cultures.

MATERIALS

Cultures. A strain of Type b *H. influenzae* (225 S) was used in all but one of the experiments, and in this a Type a strain (35 S) was employed.

Levinthal broth and agar. A stock medium is made by boiling and then filtering 10 per cent sheep-blood broth. The culture-broth is prepared by adding one part of this stock medium to three parts of plain broth. The agar medium is prepared by adding

one part of this broth to an equal amount of 4 per cent nutrient agar. The details of the preparation have been given in a previous paper (Pittman, 1931).

Serum. The serum was obtained from defibrinated normal rabbit blood. The rabbit was bled immediately preceding the set-up of the experiment.

Lake red blood cell solution. Rabbit erythrocytes were washed free of serum and laked with distilled water. The stroma was removed by centrifugation. A fresh solution was prepared for each experiment.

Yeast extract. This was prepared according to the method described by Thjötta and Avery (1921).

THE GROWTH OF HEMOPHILUS INFLUENZAE IN LEVINTHAL BROTH

Experiment 1. In this experiment two sets of small Pyrex tubes, 7 tubes in each set, were employed. The total capacity of each tube was approximately 4 cc. Into each tube was placed 0.5 cc. of Levinthal broth, and then 0.05 cc. of a broth culture diluted with broth in such a manner that the final dilution of the culture in the different tubes of each set ranged approximately from 10^{-2} to 10^{-8}. The tubes of one set were plugged with cotton and placed in the incubator at rest; those of the other set were sealed with paraffined corks, placed in a rotating machine and incubated while in motion. At the end of twenty-four hours, the amount of growth which had occurred was determined by the relative turbidity of the medium and also by the growth which resulted from a transfer of a loopful from each of the tubes to a Levinthal agar plate.

The results given in table 1 show that when the cultures were kept at rest during incubation, no growth occurred in the tubes inoculated with less than 5,000 bacteria, whereas, when the cultures were kept in motion, growth took place in all tubes, even 5 bacteria being able to initiate growth. Furthermore, the growth which occurred in the tubes at rest was less luxuriant than that which occurred in the corresponding tubes which were rotated.

Since, in the rotated tubes, the broth was continuously mixed with the relatively large amounts of air which was sealed in the

tubes, it appeared that aeration was the influential factor in bringing about the increase in growth. In each 4 cc. tube there was approximately 3.2 cc. of air, 0.8 cc. of space being occupied by the broth culture and the cork. The color changes which occurred during the growth of the bacilli in the presence of rabbit blood under the conditions of experiment 1 showed that this amount of air shaken with 0.5 cc. of blood would keep the blood oxygenated until a luxuriant growth occurred.

Undiluted and varying dilutions of the blood were employed. After incubation it was noted that the cultures which had been held at rest

TABLE 1

Influence of motion on the growth of Hemophilus influenzae in Levinthal broth

FINAL DILUTION OF CULTURE	APPROXIMATE NUMBER OF BACTERIA INOCULATED	0.5 CC. LEVINTHAL BROTH—INCUBATION	
		At rest	In motion
10^{-2}	5,000,000	4+	5+
10^{-3}	500,000	4+	5+
10^{-4}	50,000	2+	5+
10^{-5}	5,000	+	5+
10^{-6}	500	−	5+
10^{-7}	50	−	5+
10^{-8}	5	−	+

5+ = Very luxuriant growth, medium very turbid; − = no growth, medium clear.

Transfer of a loopful from each culture which was turbid to a Levinthal agar plate produced colonies, the number of which confirmed the amount of growth as estimated by the turbidity of the medium. Transfers from the cultures which were clear produced no growth.

were of a deep cherry-red color, whereas the corresponding cultures which had been kept in motion were of a much brighter red, unless a very luxuriant growth had taken place, in which event the cultures were almost black. In addition, it was noted that if the tubes which had been rotated were allowed to stand for a few hours the hemoglobin would be reduced to the same state as in the tubes which had been incubated at rest, but on reshaking, the hemoglobin would again become oxygenated and of a bright red color.

In other experiments all of the cultures were kept at rest, and a variation in the supply of air was brought about by varying the amount of surface exposed to the air.

Experiment 2. Of Levinthal broth, 9 cc. were added to each of seven 100 cc. Erlenmeyer flasks and to each of seven test-tubes 1.6 cm. in diameter. Then, to each of the flasks was added 1 cc. of a given dilution of culture, so that the final dilution of the culture in the different flasks ranged from 10^{-2} to 10^{-8}. The test-tubes were inoculated in a similar manner. All cultures were incubated for twenty-four hours, after which the amount of growth was estimated by the turbidity of the medium. Before the readings were made, each flask culture was transferred to a test-tube of the same size as had been used for the test-tube cultures.

TABLE 2

Influence of exposure of the medium to air on the growth of Hemophilus influenzae in Levinthal broth

FINAL DILUTION OF CULTURE	APPROXIMATE NUMBER OF BACTERIA INOCULATED PER CUBIC CENTIMETER	10 CC. OF LEVINTHAL BROTH IN	
		100 cc. Erlenmeyer flask	16 cm. x 1.6 cm. test-tube
10^{-2}	5,000,000	5+	3+
10^{-3}	500,000	5+	3+
10^{-4}	50,000	5+	2+
10^{-5}	5,000	4+	2+
10^{-6}	500	3+	+
10^{-7}	50	+	−
10^{-8}	5	±	−

5+ = Very luxuriant growth, medium very turbid; − = no growth, medium clear.

In table 2 it is shown that when the medium had a relatively large surface exposed to the air, 5 bacteria per cubic centimeter initiated growth. On the other hand, when the medium had a relatively small surface exposed to the air, 500 bacteria per cubic centimeter was the smallest number which induced visible growth, and in addition the final amount of growth was less than in the corresponding culture to which the air was more accessible.

The above results indicate that the growth of *H. influenzae* in Levinthal broth is directly influenced by the extent to which the medium is aërated. The results of the following experiment indicate that it is the oxygen of the air which is the important factor.

Experiment 3. Of Levinthal broth, 50 cc. were placed into each of two 250 cc. Erlenmeyer flasks. Then the broth in each flask was inoculated with 2 cc. of a young broth culture of *H. influenzae* Type *a*. The flasks were placed side by side in the incubator. One of the flasks had been fitted with a rubber stopper holding a piece of glass tubing which extended below the surface of the medium. After the flask was placed in the incubator the tubing was connected with a tank of oxygen and a stream of the gas was slowly bubbled through the medium during the six hours that the flasks were allowed to incubate. At the end of the period it was found that while good growth had occurred in both flasks, the growth was much more luxuriant in the flask which had received oxygen than in the untreated one.

In addition to the experiments on the growth of *H. influenzae* in Levinthal broth, attempts were made to grow these organisms in other nutrient broths under conditions similar to those in experiment 1. In contrast to the results obtained with Levinthal broth, it was found that when either Fildes' pepsin digested blood broth (1920), or plain broth containing washed red-blood cells of a rabbit was used in place of Levinthal broth, it was not necessary to increase the supply of air by rotating the tubes in order to obtain growth from a small inoculum of bacteria. Furthermore, the amount of growth occurring in either of these media incubated at rest was similar to that which occurred in Levinthal broth incubated in motion.

Both the Levinthal and the other two media are prepared with plain broth. In the preparation of Levinthal medium, the broth is boiled after the blood is added, and the large coagulum which is formed is removed by filtration. In the other media the blood or blood preparations are neither coagulated nor heated above 55°C. Since the V-factor is slightly thermolabile and can be absorbed, it seemed possible that the Levinthal broth contains a smaller amount of V-substance, and that to this fact its difference in supporting growth is due.

In order to test this hypothesis, the following experiment was performed.

Experiment 4. To 9 parts of Levinthal broth was added 1 part of yeast extract, and then the growth-promoting action of the mixture was tested in the same way as in experiment 1.

In table 3, it is shown that, when the V-content of the broth was increased, a small number of bacilli was able to initiate growth during incubation at rest, and the amount of growth which occurred was almost as great as that which took place in Levinthal broth alone incubated while in motion, as shown in table 1. In the case where the cultures in the enriched broth were incubated in motion, still more luxuriant growth occurred.

It therefore seemed that the inability of a small number of bacilli to initiate growth in Levinthal broth in test-tubes at rest

TABLE 3

Influence of yeast extract (V) on the growth of Hemophilus influenzae in Levinthal broth

FINAL DILUTION OF CULTURE	APPROXIMATE NUMBER OF BACTERIA INOCULATED	LEVINTHAL BROTH AND YEAST EXTRACT INCUBATION	
		At rest	In motion
10^{-2}	10,000,000	5+	6+
10^{-3}	1,000,000	5+	6+
10^{-4}	100,000	5+	6+
10^{-5}	10,000	5+	6+
10^{-6}	1,000	4+	6+
10^{-7}	100	2+	6+
10^{-8}	10	+	5+

Each tube contained 0.5 cc. of the mixture.

6+ = Very profuse growth, broth very cloudy; + = scant growth, broth almost clear.

was because an insufficient amount of growth factor V was present. Furthermore, since the deficiency of the medium was not evident when it was well aërated, either by shaking or by exposing a relatively large surface to the air, it is obvious that a large supply of air or oxygen will enable a small number of bacilli to grow in the presence of less V than would otherwise be necessary. On Levinthal agar, where a large supply of air is available, growth is always as good, and frequently better, than on rabbit blood agar or Fildes' pepsin digested blood agar.

THE EFFECT OF FRESH SERUM ON THE GROWTH-PROMOTING ACTION OF HEMOGLOBIN SOLUTION

When influenzae bacilli are grown in suspensions of intact rabbit red-blood cells and in hemoglobin solutions obtained from an equal number of laked red cells, the amount of growth occurring in each case is about the same. If, however, fresh rabbit serum is added to each medium, the amount of growth occurring in the presence of the hemoglobin solution is much less than in the medium containing whole red cells.

Terada (1922) has also shown that when fresh serum is added to laked red cells, the growth-promoting substance of the cell, unprotected by the cell membrane, is quickly destroyed. Knorr (1924) found that it is the V-factor of the cell solution which is destroyed by fresh serum. Knorr and Gehlen (1925) have shown that the V-factor of lemon juice is also destroyed in the presence of fresh serum.

The fact that the V-factor in a laked red cell solution is especially susceptible to the action of fresh serum enabled us to observe the influence of aëration on the growth of *H. influenzae* in a menstruum in which the V-factor is, at least partially, rendered inert.

Experiment 5. To varying dilutions of fresh rabbit serum was added laked red-cell solution in an amount sufficient to produce a 1:200 final dilution; the volume in which it was added was so small that the concentration of the serum was not materially affected, 0.04 cc. of a 1:2 dilution being added to 4 cc. of serum or serum dilution. Then the growth-promoting action of each mixture was tested in the same manner as in experiment 1. Two control sets of tubes were included with each series—one set containing the laked red-cell solution without serum, and the other set containing plain broth. The tubes were allowed to incubate for twenty-two hours, after which a loopful from each tube was subcultured on Levinthal agar.

The results of the experiment given in table 4 show that good growth took place in each of the cultures containing the laked red-cell solution with no serum, and there was no apparent difference in the initiation and the amount of growth due to the mode of

incubation. However, in the cultures containing the same amount of laked red-cell solution, but to which serum had been added, growth was inhibited in the tubes containing larger amounts of the serum. When the tubes were kept at rest, growth occurred only when the serum was present in a concentration not greater than 1:10, but when the tubes were kept in motion,

TABLE 4

Influence of aëration on the growth of Hemophilus influenzae Type b in the presence of fresh rabbit serum

FINAL DILUTION OF CULTURE	APPROXIMATE NUMBER OF BACTERIA INOCULATED	PLATE SUBCULTURES FROM TUBES BEFORE INCUBATION	MODE OF INCUBATION	LAKED RED CELL SOLUTION					PLAIN BROTH
				FRESH RABBIT SERUM				Saline	
				Undiluted	1:2	1:5	1:10		
10^{-2}	10,000,000	+++	At rest	−	−	+++	++++	++++	−
10^{-3}	1,000,000	++		−	−	−	++++	++++	−
10^{-4}	100,000	+		−	−	−	++++	++++	−
10^{-5}	10,000	+		−	−	−	++++	++++	−
10^{-6}	1,000	±		−	−	−	++++	++++	−
10^{-7}	100	−		−	−	−	++++	++++	−
10^{-8}	10	−		−	−	−	++++	++++	−
10^{-2}	10,000,000	+++	In motion	−	±	++++	++++	++++	−
10^{-3}	1,000,000	++		−	−	++++	++++	++++	−
10^{-4}	100,000	+		−	−	++++	++++	++++	−
10^{-5}	10,000	+		−	−	++++	++++	++++	−
10^{-6}	1,000	±		−	−	++++	++++	++++	−
10^{-7}	100	−		−	−	+++	++++	++++	−
10^{-8}	10	−		−	−	±	++++	++++	−

Each tube contained 0.5 cc. of a mixture.

++++; +++; ++; +; ±; − = Very many; many; moderate; few; very few; or no colonies, which grew from the transfer of one loopful of a culture to Levinthal agar.

growth occurred when the concentration of serum was 1:5. Although bacteria were recovered from the tube at rest containing serum in a 1:5 dilution which was inoculated with 10,000,000 bacteria, the number of colonies on the plate was no greater than the number which had developed from a subculture made before incubation. In the plain broth, the bacteria not only failed to grow, but they actually died.

This experiment suggested that the failure of growth in the presence of the higher concentrations of serum was due to a deficiency in V-substance, this substance in the red cell solution having been partially destroyed or inhibited by the serum, and, further, that this deficiency can be at least partially compensated for by increasing the degree of aëration. An experiment was therefore planned to determine whether growth would take place in the presence of higher concentrations of serum when the supply of the V-substance is increased.

Experiment 6. This experiment differs from the preceding one only in that each tube contained a 1:10 final dilution of yeast extract, the undiluted serum now being diluted 9:10, but in the diluted serum the yeast extract was used as a part of the diluent. The same serum and the same culture dilutions were used in each experiment.

The results recorded in table 5 show that the additional amount of the V-substance enabled the bacilli to initiate growth in the presence of higher concentrations of fresh rabbit serum. In the case where the tubes were incubated at rest, good growth occurred in each tube containing the 1:5 dilution of serum, whereas in the same dilution of serum with less V-factor the bacteria died. In the case where the tubes were incubated while in motion, growth occurred in every tube except the three containing the 9:10 dilution of serum which were inoculated with the three highest dilutions of the culture.

Although it appeared that the effect of shaking the cultures was due to increased aëration, thereby enabling the bacteria to grow in the presence of serum containing an inadequate amount of the V-substance, it seemed necessary to eliminate any possibility that the shaking might have caused some change in the serum itself which destroyed its inhibitory action. This doubt was removed by the results of the following experiment.

Experiment 7. Varying dilutions of fresh rabbit serum containing hemoglobin solution were added to small test-tubes as in experiment 5, table 4. But, before inoculating the mixtures with bacteria, half of the tubes were sealed, placed in the rotating machine and incubated while in motion; the other half were placed in the ice box. After

twenty-two hours the tubes were removed from the incubator and ice box and inoculated with bacteria. They were then incubated at rest for twenty-two hours, after which subcultures were made to Levinthal agar to determine the presence of growth. It was found that the initiation and the amount of growth which took place in each of the

TABLE 5

Influence of yeast extract (V) on the growth of Hemophilus influenzae Type b in the presence of fresh rabbit serum

FINAL DILUTION OF CULTURE	APPROXIMATE NUMBER OF BACTERIA INOCULATED	MODE OF INBUBATION	YEAST EXTRACT					
			LAKED RED CELL SOLUTION					Saline
			FRESH RABBIT SERUM				Saline	
			9:10	1:2	1:5	1:10		
10^{-2}	10,000,000	At rest	−	−	++++	++++	++++	++++
10^{-3}	1,000,000		−	−	++++	++++	++++	++++
10^{-4}	100,000		−	−	++++	++++	++++	−
10^{-5}	10,000		−	−	++++	++++	++++	−
10^{-6}	1,000		−	−	++++	++++	++++	−
10^{-7}	100		−	−	++++	++++	++++	−
10^{-8}	10		−	−	++++	++++	++++	−
10^{-2}	10,000,000	In motion	++++	++++	++++	++++	++++	++++
10^{-3}	1,000,000		++++	++++	++++	++++	++++	+++
10^{-4}	100,000		++++	++++	++++	++++	++++	++++
10^{-5}	10,000		++++	++++	++++	++++	++++	+++
10^{-6}	1,000		−	++++	++++	++++	++++	+++
10^{-7}	100		−	++++	++++	++++	++++	−
10^{-8}	10		−	++++	++++	++++	++++	−

Each tube contained 0.5 cc. of a mixture.

++++; +++; ++; +; − = Very many; many; moderate; few; or no colonies, which grew from the transfer of one loopful of a culture to Levinthal agar.

tubes which had been incubated in motion before inoculation was the same as that which took place in corresponding tubes which had been previously held in the ice box.

From this experiment it is apparent that the better growth which occurs in the presence of serum when the cultures are kept in motion during incubation is not due to some influence of shaking on the serum, as the bacteria grew no better in the tubes

that had been shaken before inoculation than in the tubes that had not been previously shaken. It is also apparent that complement plays no significant rôle in preventing the growth of the bacteria in the presence of fresh rabbit serum, since shaking the serum for twenty-two hours at 37°C. would destroy at least the greater part of it.

SUMMARY

A definite relation exists between the amount of the V-substance and the amount of air required for the growth of *Hemophilus influenzae* Type b in certain media. In Levinthal broth with a relatively small surface exposed to the air, a small number of the bacilli are unable to initiate growth. Growth can be initiated by a few bacteria, however, if either the supply of air or the amount of the V-substance is increased. The supply of air was increased by rotating test-tube cultures during incubation or by exposing a relatively large surface of the broth to the air. The increase in the V-substance was brought about by the addition of yeast extract to the broth.

The same relation between the supply of air and the supply of V exists when the bacteria are grown in the presence of fresh rabbit serum, which apparently destroys or inhibits the action of the V-substance. Attempts were made to grow the bacteria in mixtures containing laked red-cell solutions and varying dilutions of fresh rabbit serum. The mixtures were placed in duplicate sets of tubes, and during incubation one set of tubes was kept at rest and the other in motion. In both cases no growth occurred in the higher concentration of the serum, and this effect is considered to be due to the inactivation of at least part of the V-substance by the fresh serum. In the cultures which were kept well aërated by shaking, however, growth occurred in the presence of greater concentrations of serum than in cultures which were kept at rest. The better aëration apparently enabled the bacteria to grow in the presence of an amount of the V-factor insufficient to promote growth with less air. Moreover, by increasing the amount of V-substance and incubating the cultures at rest, growth took place in the same concentration of serum as in cultures containing less V which were incubated while in motion.

It has been clearly demonstrated that when certain media are kept well aërated, the bacilli are able to grow in the presence of less V than would otherwise be required, and, *vice versa*, if the V-factor is increased, growth will occur with less aëration. The X-factor was present in all cultures.

CONCLUSIONS

A small number of *Hemophilus influenzae* Type b bacilli can only initiate growth in certain media containing a small amount of V-factor if the culture is kept well aërated. On the other hand, if the amount of the V-factor is increased, growth can be initiated in the presence of less air. It seems, therefore, that the V-factor as well as the X-factor plays a part in the oxidation processes of *H. influenzae*.

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SOME OBSERVATIONS ON THE GERMICIDAL EFFICIENCY OF CHLORAMINE-T AND CALCIUM HYPOCHLORITE

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Although chlorine compounds having disinfectant properties have been used for over a century, the fundamental factors which determine their germicidal efficiencies are not yet well understood and are certainly not generally appreciated. New types of chlorine disinfectants with distinct germicidal properties have come upon the market. The concentrations of these compounds are customarily expressed in parts per million available chlorine and are determined by simple titration or colorimetric tests but the analyses do not give an accurate idea of the germicidal activity of the solutions tested. This fact has, perhaps, not been adequately appreciated by health authorities, as residual chlorine concentrations that should be maintained for a desired germicidal efficiency are frequently stipulated without qualification. In some sterilizing procedures a quick germicidal action is desired, in others, a mild but prolonged action may be preferred. The technical applications and uses of germicidal chlorine compounds in sanitation are numerous and varied, and with proper appreciation of the nature of the disinfectant and of the factors affecting its action more efficient and dependable applications may be made.

The authors' primary object was to obtain information on the effect of temperature, reaction (pH) and concentration on the germicidal efficiency of some chlorine compounds.

For the purpose of this study bacterial spores appear to be particularly suitable. Some advantages may be suggested; higher concentrations of the disinfectant can be employed with

resultant greater accuracy in concentration control; studies over wider ranges of temperature and reaction are possible than could be employed in the case of non-spore-forming bacteria; a test organism of practically uniform resistance can be readily made up from day to day in suspensions of almost the same numbers. The destruction of vegetative cells by chlorine compounds is so rapid that accurate determination of survivors is subject to considerably greater error than is the case with spores. Although the destruction of spores is not required in many practical disinfection procedures, the use of a spore suspension in disinfection studies has distinct advantages in minimizing difficulties in experimental techniques associated with the evaluation of environmental factors studied.

Because of the state of purity in which chloramine-T may be obtained on the market and the ease with which the desired concentrations may be duplicated, this compound was chosen for the first studies. Test solutions of desired concentration were made by dissolving previously determined weighed amounts of choramine-T in distilled water. In all cases the concentrations were expressed in parts per million available chlorine. The reactions (pH) were determined with the glass electrode.

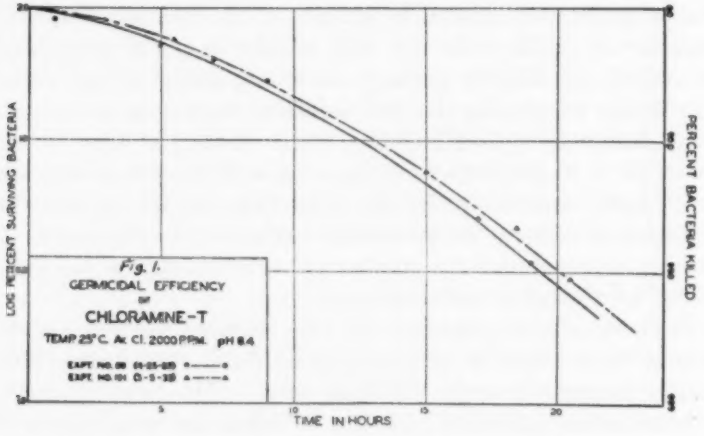
The bacterial spores used in this study were from a culture which had previously been employed in disinfection experiments by Levine, Buchanan, and Lease (1927) and by Myers (1929). A detailed description of the organism, *Bacillus metiens* (*nov. sp.*) will appear in a forthcoming publication. The spore preparation was obtained by scraping off the surface growth from nutrient agar (Difco) in Kolle flasks, drying, grinding to a fine powder in an agate mortar and thoroughly mixing with sterile powdered lactose. Some objection might be made on the ground that the test solutions are practically free from organic matter while this is rarely the case in practice. However, had blood serum been added, as has often been done in disinfection studies, neither the reaction nor chlorine concentration would have remained constant and for the purposes of this study relative constancy in these factors is essential.

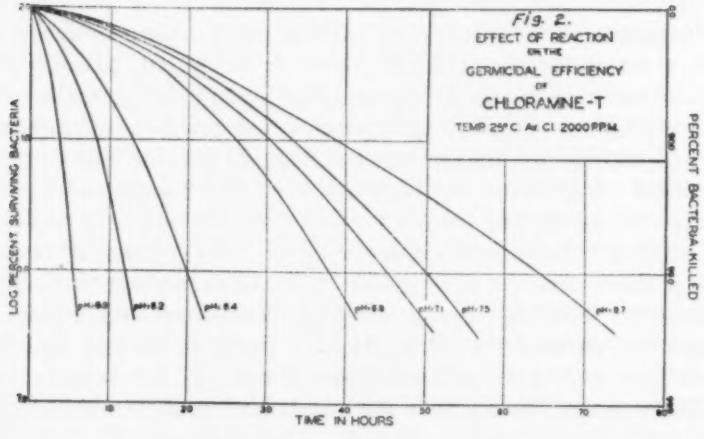
The bacterial spore suspensions employed in the disinfection

experiments were obtained by weighing out approximately 0.2 gram of the spore preparation, suspending it in sterile tap water and filtering through a fine grade of filter paper to remove clumps. Dilutions from the filtrate were made so that about 10,000,000 viable spores were placed in 100 ml. of the test solution. The number of viable cells per unit weight of spore preparation decreased only slightly during a six-month period of observation. The flasks containing the test solutions were held at constant temperature in a DeKhotinsky water bath. At desired time intervals, 5 cc. portions were removed and introduced into dilution blanks containing slightly more than enough sodium thiosulphate to destroy the germicidal action of the chlorine in the sample removed and the number of surviving spores was determined by plating on nutrient agar.

For comparative purposes, the time required to effect a reduction of 99 per cent in the numbers of viable spores was chosen and is designated as the "killing time." The need for such a determination instead of "death rate" when the death rate is not logarithmic, has been pointed out previously by Levine and Buchanan (1928). A typical experiment is shown in figure 1. At a concentration of 2,000 p.p.m. available Cl, pH 6.4 and temperature 25°C., the killing time was found to be approximately twenty hours. Similar experiments were carried out at the same concentration and temperature but with the reaction (pH) varied. Control experiments in the absence of the disinfectant in the range pH 5.8 to 9.3, showed the test organisms to be unaffected even after two weeks exposure at 25°C. The average of two or three check runs at each reaction (pH) chosen are summarized in figure 2. The pronounced effect of reaction on killing time is apparent, particularly below pH 7.0. Owing to the fact that the solutions were not buffered, those above pH 7.0 became less alkaline during the course of the experiment, probably due in part to absorption of carbon dioxide. Had this not occurred, the killing times would presumably have been longer with solutions above pH 7.0. The pH of the other solutions (more acid than pH 7.0) was found to be practically constant throughout the experiment. The killing times obtained at three concentrations,

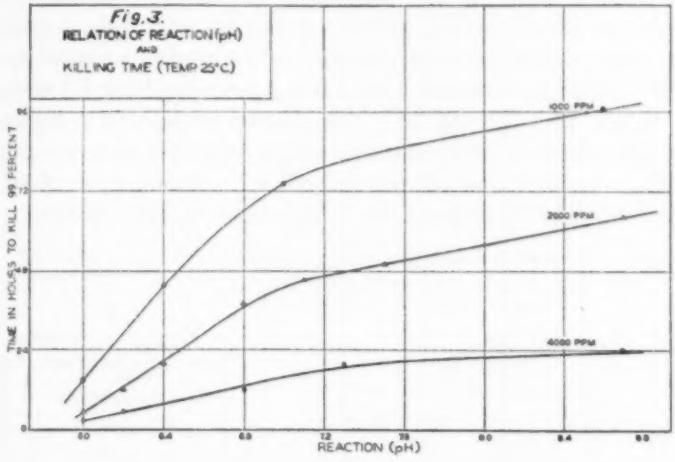
plotted against reaction, are shown in figure 3. In the range pH 6.0 to 7.0 the curves tend to approach straight lines (and this relationship is more apparent with the higher concentrations).

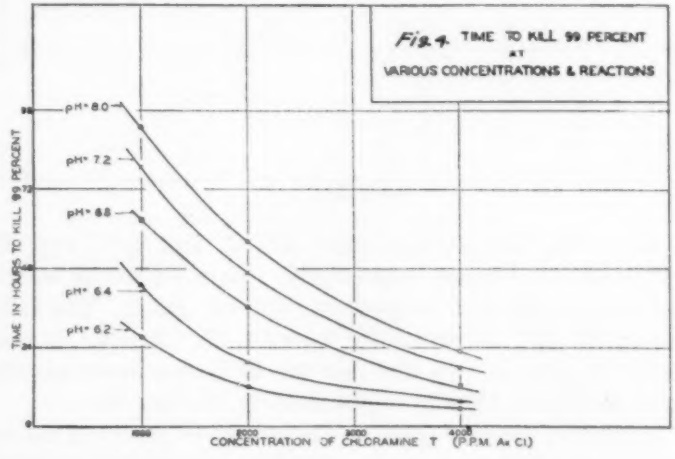




From figure 3 data can be obtained to show the effect of concentration on killing time at a constant reaction (pH). These relationships are shown in figure 4. The effect of doubling the concentration at constant reaction (pH) is apparent. At pH 6.2,

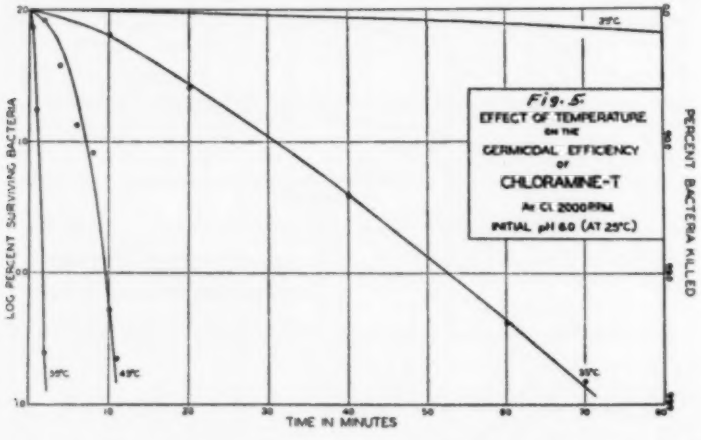
for example, the killing times are 27, 12 and 5.4 hours for the 1,000, 2,000, and 4,000 p.p.m. concentration respectively. In this case, the reduction in killing time is about 55 per cent for each doubling





of the concentration. Similar comparisons from the other curves show killing time reductions of 40 to 65 per cent when the concentration of available chlorine is doubled.

The effect of temperature on killing time at a given concentration and initial pH (determined at 25°C.) was also studied. The reactions employed were pH 6.0 and pH 8.7. Control experiments with the test organisms in buffer solutions in the absence of chloramine-T at pH 9.3 and 5.8 and temperature of 55°C. did not disclose a disinfecting action during a period of six hours. Results obtained with 2,000 p.p.m. available Cl and pH 6.0, at 25°, 35°, 45° and 55°C., are shown in figure 5. Figure 6 shows the marked effect of temperature on killing time for initial reactions (at 25°C.) of pH 6.0 and pH 8.7. Thus, a rise of 10°C. (at initial pH 6.0) reduces the killing time by approximately 82



per cent. The killing times at 25°, 35°, 45° and 55°C. were 324, 53, 9.7 and 1.8 minutes respectively. With an initial reaction of pH 8.7, the effect of temperature is not so great. The killing times at 25°, 35°, 45° and 55°C. were 64, 17, 5.5 and 1.55 hours respectively (fig. 6). In this case the 10°C. rise in temperature reduces the killing time by approximately 71.5 per cent.

The effect of reaction (pH) on the germicidal power of calcium hypochlorite solutions was also noted, employing the technique with spores described above. The results were similar to those obtained with chloramine-T in that increasing acidities increased germicidal efficiency. Some results are given in table 1. (The

hypochlorite employed was one of the commercial types sold in powder form containing high concentrations of available chlorine.)

It is of interest to note the following. A solution of calcium

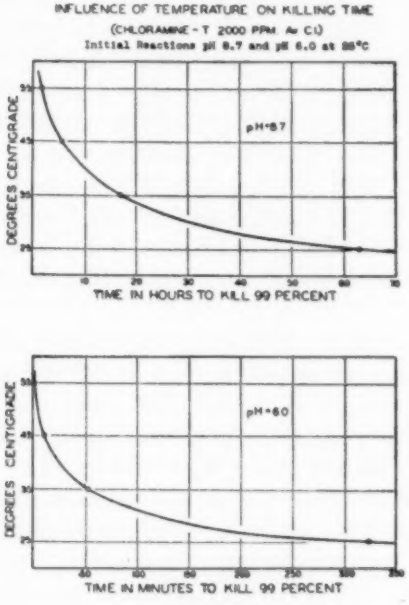

INFLUENCE OF TEMPERATURE ON KILLING TIME
(CHLORAMINE - T 2000 PPM Av Cl)
Initial Reactions pH 8.7 and pH 6.0 at 25°C
DEGREES CENTIGRADE
pH=8.7
TIME IN HOURS TO KILL 99 PERCENT
DEGREES CENTIGRADE
pH=6.0
TIME IN MINUTES TO KILL 99 PERCENT

FIG. 6

TABLE 1

Germicidal efficiency of calcium hypochlorite, temperature 25°C.

CONCENTRATION P.P.M. AVAILABLE Cl	REACTION (pH)	TIME TO DESTROY 99 PER CENT BACTERIAL SPORES
1000	11.3	64 minutes
1000	7.3	Less than 20 seconds
100	10.4	70 minutes
20	8.2	5 minutes

hypochlorite containing 1,000 p.p.m. available chlorine had a reaction of pH 11.3 and required 64 minutes to kill 99 per cent of the exposed spores. Addition of HCl to this solution, bringing the reaction to pH 7.3, reduced the killing time to less than twenty

seconds. The marked influence of reaction on germicidal efficiency of hypochlorite is evident.

Diluting the original hypochlorite solution with distilled water ten times to an available chlorine content of 100 p.p.m. did not appreciably prolong the killing time which was now seventy minutes as compared with sixty-four minutes for the undiluted (1,000 p.p.m. solution) but the reaction of the diluted solution was pH 10.4 as compared with pH 11.3 of the original, again indicating the marked effect of reaction. Further dilution fifty fold to 20 p.p.m., coupled with addition of HCl which brought the reaction to pH 8.2, resulted in a killing time of only five minutes. Thus it is evident that "available chlorine" is not a dependable index of the germicidal efficiency of calcium hypochlorite if the reaction is not constant.

The fact that acids added to hypochlorites increase germicidal efficiencies was noted in 1890 by Nissen (1890), and he attributed this effect to nascent chlorine. The view now generally held, that hypochlorous acid is the germicidal agent, was first proposed in 1904 by Andrewes and Orton (1904). These investigators stated that when hypochlorites are acidified with a weak acid, hypochlorous acid is liberated. They prepared the pure acid and found it to have an intense germicidal action. The percentage of hypochlorite present in the form of hypochlorous acid at various reactions (pH) has been calculated by Holwerda (1928) and his bacteriological studies have shown the increasing germicidal power to run parallel with an increase in the HOCl concentration.

SUMMARY

A study was made of the effect of temperature, concentration and reaction on the germicidal efficiency of chloramine-T. Bacterial spores were found to be particularly suitable for the purpose of this study and comparisons were based on the time required to effect a reduction of 99 per cent of the exposed organisms. The reactions (pH) of the solutions were determined with the glass electrode.

For a given concentration of available chlorine as chloramine-T, a rise of 10°C. resulted in a decrease in the killing time of about 82

per cent if the initial reaction was pH 6.0 and approximately 71.5 per cent at pH 8.7. Doubling the disinfectant concentration at a given initial reaction and constant temperature reduced the killing time about 40 to 60 per cent. The pH range studied was from 6.0 to 8.8, and it was found that increasing the acidity markedly reduced the killing time.

Some experiments were also performed with hypochlorites. Here again, pH seemed to be the greatest single factor affecting the germicidal activity of these compounds against spores of the test organism.

"Available chlorine" was not found to be a direct measure of the germicidal efficiency of the calcium hypochlorite studied. A solution containing 1,000 p.p.m. available chlorine was only slightly more germicidal than the same solution diluted with distilled water to 100 p.p.m. (the reaction was changed by dilution from pH 11.3 to pH 10.4), and very much less efficient than 20 p.p.m. of the same disinfectant at a reaction of pH 8.3. The killing times of the various concentrations of calcium hypochlorite were sixty-four minutes, seventy minutes and five minutes for solutions of 1,000 p.p.m. at pH 11.3, 100 p.p.m. at pH 10.4 and 20 p.p.m. at pH 8.3, respectively.

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PHYSIOLOGICAL STUDIES ON RHIZOBIUM

IV. UTILIZATION OF CARBONACEOUS MATERIALS[1]

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Although extensive experiments have been conducted with the legume root-nodule bacteria, no acceptable theory has been advanced to explain the specificity of the organisms in nodule production. Different species may show similar morphological and cultural characteristics, and yet be unable to bring about nodulation upon any plants except their particular hosts. Little is known of the materials presumably furnished the organisms by the host plant during the period of the symbiotic growth, nor is it known whether or not the compounds supplied by the plants of the same cross-inoculation groups are similar.

It has been shown that the grouping of legume bacteria on the basis of cross-inoculation is similar to that indicated by serological reactions in cross-agglutination. The suggestion has also been made that certain materials given off by the roots of leguminous seedlings at a certain stage of development stimulate the formation of nodules. Hence, attempts have been made to show a relationship between the composition of plant roots and tops and the ability of certain organisms to bring about nodule formation. It has been shown further that the different species of Rhizobium differ in their ability to utilize carbonaceous materials. In view of all these facts it seems probable that studies of carbohydrate utilization or dissimilation may yield information of value in explaining the specificity of the several species.

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[2] The authors are indebted to Dr. P. E. Brown for the suggestions and criticisms offered in the course of this work and in the preparation of the manuscript.

It seems further that the relative utilization of nitrate and ammonium compounds by the various organisms may suggest an explanation of the mechanism of nitrogen fixation. A more or less commonly accepted theory of nitrogen fixation is that the phenomenon occurs through the reduction of elemental nitrogen. Perhaps, under certain conditions, the organisms are able to utilize molecular nitrogen as a hydrogen acceptor in the dissimilation of carbonaceous materials.

It is obvious that studies on the utilization of energy materials by Rhizobium will aid in the solution of the problems involved in inoculation and nitrogen fixation. Since the organisms of this group grow aerobically, using molecular oxygen chiefly as a hydrogen acceptor, the amount of oxygen consumed should measure the carbohydrate utilized.

The experimental evidence concerning the utilization of carbonaceous materials by Rhizobium is rather fragmentary. Many of the observations reported in the literature are of a more or less incidental nature, having been made in connection with a study of some other problem. The fact that a more complete fund of knowledge of the utilization of carbonaceous materials by Rhizobium might contribute materially toward a solution of certain complex problems pertaining to these organisms, has been pointed out. In the light of these facts, it seemed desirable that a systematic study of the utilization of carbonaceous materials by Rhizobium be undertaken.

The investigations reported here have been concerned with (1) the rate and extent of oxygen consumption by the root-nodule organisms grown on various carbonaceous materials, and (2) the influence of nitrate and ammonia nitrogen on respiratory activity.

METHODS

Respiration methods have been used to study the utilization of various carbonaceous materials by two species of the root-nodule bacteria. The consumption of oxygen was measured by the use of blood gas micromanometers of the type described and used by Warburg (1926) in his investigations concerning the respiration of tumor tissue. Burk and Milner (1932) and Walker,

Anderson and Brown (1933) have pointed out the value of this apparatus and technique for the study of bacterial respiration. Oxygen consumption as determined by the manometric method is much more accurate than when determined by ordinary gas analysis, as is pointed out by Burk and Milner (1932) and by Georgi and Wilson (1933).

The manometer vessels were prepared for each experiment by placing 0.3 cc. 2M KOH in the small container in the center of the cup, and 1.0 cc. of a double concentration of the substrate plus 1.0 cc. of the inoculum in the outer compartment. The vessels were immediately connected with the manometers and placed on the water bath where they were allowed to attain a temperature equilibrium before being closed to start the experiment. The temperature of the bath was maintained at 28.0°C. ±0.01°. The manometers were shaken at the rate of from 90 to 95 oscillations per minute, with an amplitude of approximately 3 cm.

PRELIMINARY EXPERIMENTS

Preparation of inoculum

The inocula first used in the respiration experiments reported here were prepared with organisms grown on a medium of the following composition: Glucose, 10.0 grams; K_2HPO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.1 gram; $CaCO_3$, 3.0 grams; Bacto-yeast-extract, 0.5 gram; Agar, 15.0 grams; Distilled water, 1000.0 cc. Later, the agar was omitted and the inocula prepared with organisms grown in the resulting liquid medium.

Some preliminary experiments were conducted to determine the best method for the preparation of inocula. First, the organisms were washed from the surface of an agar slant with a sterile 0.425 per cent NaCl solution and this suspension used as the inoculum. The amounts of oxygen consumed by these organisms on various substrates are given in figure 1. Curve I shows the consumption of oxygen on a substrate which provided only the mineral salts with no carbohydrate added. Curve II shows the results when 1 per cent glucose was included in the medium. In the third case, 1 per cent mannitol was present. The consumption of oxygen in the control flask amounted to 71.4 per

cent and 66.5 per cent, respectively, of the consumption observed in the two cases when a suitable carbohydrate was added to the substrate. This large consumption of oxygen in the culture without added energy material could hardly be attributed to the usual endogenous respiration of the cells but rather, it was thought that it was the result of the considerable amounts of carbonaceous and nitrogenous compounds carried over in the inoculum. Proceeding on this assumption, a second inoculum

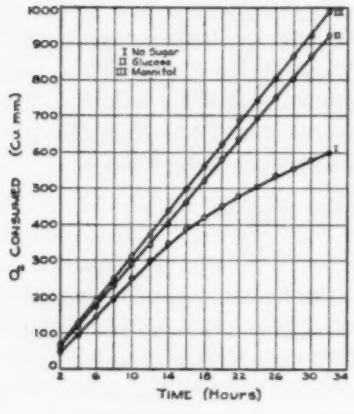

FIG. 1

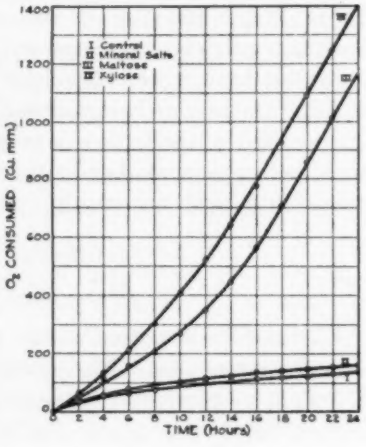

FIG. 2

FIG. 1. OXYGEN CONSUMPTION BY UNWASHED CELLS OF *Rhizobium meliloti* IN MEDIA WITH AND WITHOUT ADDED CARBONACEOUS COMPOUNDS

FIG. 2. OXYGEN CONSUMPTION BY *Rhizobium meliloti* WITH AND WITHOUT CARBONACEOUS MATERIAL ADDED TO THE MEDIA

was prepared by centrifuging the suspension of organisms obtained from the agar slant, and subsequently washing them twice in sterile salt solution. A suspension of these cells was then used as the inoculum in a second experiment. The amounts of oxygen consumed during a twenty-four-hour period are shown in figure 2. Curves I and II, respectively, show the respiratory activity of the cells when suspended in the salt solution and on a substrate which provided the usual mineral salts but no carbon source. In each

case, the consumption of oxygen proceeded steadily but very slowly throughout the duration of the experiment. When 1 per cent l-xylose was included in the substrate the consumption of oxygen increased greatly as shown by curve IV. When 1 per cent maltose was added as a source of energy (curve III), a similar increase in the respiratory activity of the organisms occurred.

In all subsequent work, the inocula were prepared by centrifuging the organisms and washing them in salt solution. In some of the later work, the organisms were grown in a liquid glucose-yeast-extract medium then centrifuged and washed in the usual way. No difference in respiratory activity was observed between the organisms grown in a liquid medium and those grown on the surface of an agar slant.

Agreement between duplicates

All of the experiments reported were conducted in duplicate, and the data presented, unless otherwise stated, represent averages of the various duplicate determinations. In order to show the degree of agreement that may be expected between duplicates, the data of a typical experiment are presented in figure 3. Curves I and II represent the amounts of oxygen consumed when the organisms were suspended in the salt solution. Curves III and IV show the oxygen consumption when the substrate provided the mineral salts of the medium with no carbohydrate added. Curves V and VI show the volume of oxygen consumed when 1 per cent mannitol was added to the medium. These results illustrate the remarkable agreement between duplicates obtainable in respiration experiments of this type, and it seems, would justify the use of averages in presenting the data.

RESULTS

The utilization of carbonaceous materials by Rhizobium meliloti

The amounts of oxygen consumed by the organisms when grown in media containing the various carbonaceous materials were employed as a criterion of the suitability of these materials. The total amounts of oxygen consumed by the alfalfa root-nodule organisms during a period of twenty-four hours in media con-

taining 1 per cent of the various carbonaceous compounds are given in table 1. Determinations were made with both sodium nitrate and ammonium chloride as sources of nitrogen, these compounds being added in sufficient quantities to provide 100 p.p.m. of nitrogen in all media. The amounts of oxygen consumed in the various substrates are directly comparable, one with another, within each series, but are not comparable between different series due to unavoidable variations in the size of

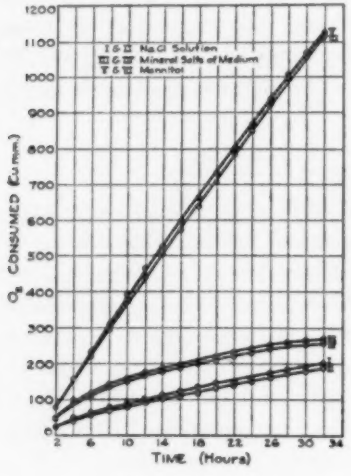

FIG. 3

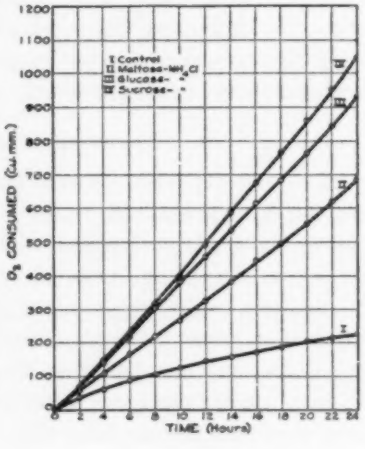

FIG. 4

FIG. 3. AGREEMENT BETWEEN DUPLICATE DETERMINATIONS OF OXYGEN CONSUMPTION BY *Rhizobium meliloti*

FIG. 4. OXYGEN CONSUMPTION BY *Rhizobium meliloti* IN MEDIA CONTAINING VARIOUS CARBONACEOUS COMPOUNDS

inoculum used in the different series of experiments. In series I, III, and IV, tests with glucose were included along with those on the other carbonaceous materials, in order to provide a basis for comparison. A test with glucose was not included in series II, hence a direct comparison cannot be made for the carbohydrates of this series against glucose. A comparison can be made, however, between the various carbohydrates of the series.

Figure 4 shows the course of oxygen consumption by *Rhizobium*

meliloti in a typical experiment. Curve I represents the amount of oxygen consumed by the cells when suspended in the salt solution with no energy material added. Curves II, III, and IV,

TABLE 1

Oxygen consumption by Rhizobium meliloti in nitrate and ammonium media with different carbonaceous compounds

CARBON SOURCE	NITRATE MEDIA WITH 100 P.P.M. OF NITROGEN		AMMONIUM MEDIA WITH 100 P.P.M. OF NITROGEN	
	C.mm. O_2 consumed	Compared with glucose*	C.mm. O_2 consumed	Compared with glucose*
Series I:				
Glucose	585.8	100	931.5	100
Mannitol	552.6	93	903.0	96
Sucrose	524.1	86	1049.4	117
Maltose	474.7	76	683.9	65
Series II:				
Xylose	550.8		1061.8	
Rhamnose	710.2		903.6	
Mannose	808.9		1129.2	
Inulin	267.0		467.6	
Series III:				
Glucose	720.0	100	1040.6	100
Arabinose	837.1	128	1450.1	165
Galactose	939.1	152	1330.5	146
Lactose	553.9	61	704.6	47
Raffinose	680.6	91	899.9	78
Series IV:				
Glucose	751.9	100	1229.4	100
Inositol	646.9	72	877.6	60
Erythritol	777.3	106	815.8	58
Dulcitol	577.5	53	881.4	61
Sorbitol	644.7	71	862.0	59

* Calculated in terms of the increase in oxygen consumption on the various carbonaceous materials over that which occurred in the control culture with no added carbon source. Oxygen consumption in glucose media taken as 100 per cent.

respectively, represent the amounts of oxygen consumed in maltose, glucose, and sucrose media.

The relative extent of oxygen consumption in the media containing the various carbonaceous compounds, as calculated in percentage of that which occurred in the glucose medium, is

also given in table 1. These values have been calculated in terms of the increase in total oxygen consumption over that which occurred in the control culture where no carbonaceous material was added.

It is apparent from the data that *Rh. meliloti* is capable of utilizing a wide variety of carbonaceous compounds. Although a test with glucose was not made in series II, with rhamnose, l-xylose, mannose, and inulin, the relative suitability of the latter carbohydrates may be observed. Of these four compounds, the first three seemed to permit vigorous respiratory activity of the alfalfa organism. Mannose permitted somewhat higher consumption on both nitrate and ammonium nitrogen than did l-xylose and rhamnose. Inulin, the only polysaccharide included in the test, was oxidized only to a very limited extent, as indicated by the amount of oxygen consumed.

Galactose and arabinose, with both nitrate and ammonium nitrogen, permitted a considerably greater consumption of oxygen than did glucose, as is shown in table 1. Mannitol, with both forms of nitrogen, and raffinose with nitrate nitrogen, gave results similar to those obtained with glucose. Sucrose seemed slightly less suitable than glucose with a nitrate medium but showed somewhat higher results when ammonium nitrogen was provided. The influence of the nitrogen source on the consumption of oxygen will be discussed later. Maltose was somewhat inferior to glucose in each case. Lactose, as has been reported by Neumann (1902) and Temple (1916), was not readily utilized by this organism. Inositol, dulcitol, and sorbitol permitted a consumption of oxygen amounting to from 60 to 70 per cent of that which occurred with glucose. Erythritol, in a nitrate medium, was equal to glucose but was decidedly inferior when ammonium chloride constituted the only nitrogen source.

The list of carbonaceous compounds given here as decomposable by *Rh. meliloti* could doubtless be extended considerably. This is particularly true inasmuch as the organisms used in these experiments had been previously grown with glucose as the sole source of carbon. Stephenson and Stickland (1932) have recently shown that a bacterial enzyme may be adaptive, i.e., that it will

be present at the outset of the test only when the substrate being studied has been present in the culture during the previous growth of the organism. Thus, it is conceivable that the relative rates of oxygen consumption reported here might be appreciably altered by the previous culture of the organisms on a substrate other than glucose. However, in all cases in this work, the consumption of oxygen proceeded at a fairly constant rate throughout the course of the experiment. This would seem to indicate that the enzymes concerned in the oxidation of these compounds were present at the start of the experiment.

The utilization of carbonaceous materials by Rhizobium japonicum

The amounts of oxygen consumed by *Rh. japonicum* both with and without a suitable carbon source, are shown in figure 5. Curve I represents the amount of oxygen consumed in the control culture. The control, as in the previous experiments, consisted of a suspension of the washed organisms in a solution of sodium chloride with no carbon source provided. Since growth would not occur in such a medium, the consumption of oxygen represents the amount of the gas utilized in the endogenous respiration of the organisms. In a medium containing 1 per cent mannitol and 100 p.p.m. nitrogen as sodium nitrate, the consumption of oxygen increased somewhat, as is shown in curve II. The total amount, however, as compared with the utilization of oxygen which occurred in glucose and arabinose media, was relatively small. Apparently mannitol does not constitute a particularly favorable source of energy for the soybean root-nodule organisms. The amounts of oxygen consumed by this organism in a glucose-nitrate medium are shown in curve III. Glucose permitted considerably greater respiratory activity of the organisms than did mannitol. This condition might be attributed to the fact that the organisms had been previously cultured in a glucose medium. The extent of oxygen consumption by these organisms in an arabinose-nitrate medium (curve IV), however, indicates that the previous substrate had little or no influence on the activity of the organisms in this case. The amount of oxygen consumed by the organisms in the arabinose medium was very much higher

than that consumed in either glucose or mannitol media. The soybean organism is commonly considered as a slowly growing organism. Perhaps under proper conditions it might attain a rate of growth at least comparable with that of *Rh. meliloti* and the other more rapidly growing species of Rhizobium.

The extent of oxygen consumption by *Rh. japonicum* in a nitrate medium with various carbon compounds as energy sources,

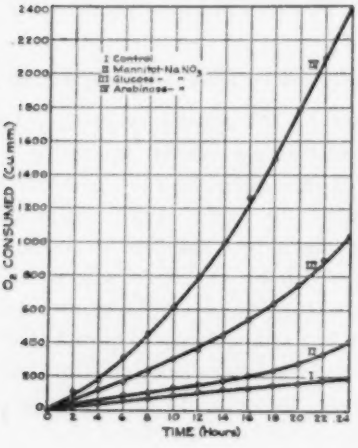

FIG. 5

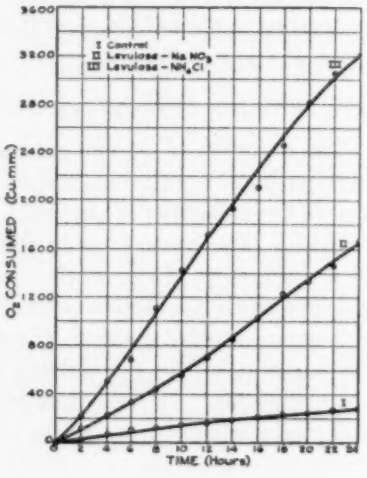

FIG. 6

FIG. 5. OXYGEN CONSUMPTION BY *Rhizobium japoncium* IN MEDIA CONTAINING VARIOUS CARBONACEOUS COMPOUNDS

FIG. 6. THE INFLUENCE OF NITRATE AND AMMONIUM NITROGEN ON OXYGEN CONSUMPTION BY *Rhizobium meliloti*

is shown in table 2. In series I and II the experiments were conducted for a period of twelve hours. Tests with both nitrate and ammonium nitrogen were included in series III. Glucose was included in each of the groups in order to provide a basis for comparison of the respiratory activity on other compounds between different series. Lactose, sucrose, and erythritol permitted only a very limited consumption of oxygen by this organism. Mannose, maltose, glycerol, and mannitol with nitrate nitrogen,

permitted a slightly increased amount of respiratory activity. Glucose, galactose, and xylose media permitted a considerably greater consumption of oxygen than did any of the compounds tested. Xylose seemed to be somewhat superior to glucose and galactose as a source of energy for the soybean organism. Arabinose with both nitrate and ammonium nitrogen, supported a greatly increased consumption of oxygen.

TABLE 2

Oxygen consumption by Rhizobium japonicum in nitrate and ammonium media with different carbonaceous compounds

CARBON SOURCE	NITRATE MEDIA WITH 100 P.P.M. OF NITROGEN		AMMONIUM MEDIA WITH 100 P.P.M. OF NITROGEN	
	C.mm. O_2 consumed	Compared with glucose*	C.mm. O_2 consumed	Compared with glucose*
Series I:				
Glucose	477.8	100		
Galactose	483.6	102		
Mannose	284.7	40		
Xylose	530.3	116		
Lactose	174.5	6		
Series II:				
Glucose	453.1	100		
Maltose	226.7	20		
Sucrose	170.4	0		
Glycerol	225.9	20		
Erythritol	129.0	0		
Series III:				
Glucose	1036.1	100	478.6	100
Mannitol	408.4	25	228.9	0
Arabinose	2345.9	255	1209.3	410

* Calculated in terms of the increase in oxygen consumption on the various carbonaceous materials over that which occurred in the control culture with no added carbon source. Oxygen consumption in glucose media taken as 100 per cent.

Table 2 also shows the relative extent of oxygen consumption by this organism on various substrates as calculated in percentage of the consumption which occurred with glucose. As in the previous case, these values have been calculated in terms of the increase in oxygen consumption on the various carbonaceous materials over that which occurred in the control culture with no added carbon source.

The ability of the soybean root-nodule organism to effect the oxidation of several more or less common carbonaceous materials varies considerably for the different compounds. Using the results obtained with glucose as a standard of comparison, it is apparent from table 2 that the oxidation of mannitol, in a nitrate medium, proceeded very slowly. In a mannitol-ammonium medium, there was no appreciable increase over the control. Arabinose permitted a very great increase in the consumption of oxygen in a nitrate medium. The high value given for the arabinose-ammonium substrate is due, not to an increase in this medium, but rather to a very considerable decrease which occurred in the glucose-ammonium substrate. This point will be discussed in the section dealing with the effect of the nitrogen source on respiratory activity. Xylose permitted a somewhat more rapid consumption of oxygen than did glucose. The superiority of arabinose and xylose as energy materials for the soybean organism has been reported by Baldwin and Fred (1927), Müller and Stapp (1925) and others. The suitability of galactose seemed approximately equal to that of glucose. Mannose was slightly superior to maltose and glycerol, but each of the three permitted only a very limited consumption of oxygen. Lactose, sucrose and erythritol permitted little or no increase in respiratory activity over that which occurred in the control flask.

Effect of nitrogen source on oxygen consumption

The relative suitability of nitrate and ammonium nitrogen for use by *Rh. meliloti* has been determined by calculating the percentage increase in total oxygen consumption on various substrates over that which occurred in the control.

With the alfalfa organism the magnitude of the increase in oxygen consumption over the control was considerably greater with ammonium media than with nitrate media (table 3). The only exception is with the lactose media where the utilization of oxygen was very low in each case. Furthermore, the difference in favor of the nitrate in this case was small and very probably would fall within the range of experimental error. The data presented in figure 6 further emphasize the superiority of the

reduced form of nitrogen for utilization by *Rh. meliloti*. The consumption of oxygen on a levulose-ammonium chloride medium was much greater than that which occurred on a levulose medium with nitrate nitrogen.

Allyn and Baldwin (1930) (1932) have recently reported that the growth of Rhizobium is partially dependent upon the oxida-

TABLE 3

The influence of nitrate and ammonium nitrogen on oxygen consumption by Rhizobium meliloti and Rhizobium japonicum

CARBON SOURCE	NITRATE MEDIA	AMMOMIUM MEDIA
Rhizobium meliloti:		
Glucose	268*	485*
Mannitol	255	494
Sucrose	222	571
Maltose	183	386
Xylose	192	355
Rhamnose	229	306
Mannose	274	406
Inulin	16	110
Arabinose	179	257
Galactose	214	227
Lactose	85	76
Raffinose	127	137
Levulose	492	1034
Glycerol	252	768
Erythritol	105	155
Dulcitol	52	164
Sorbitol	70	159
Inositol	71	162
Rhizobium japonicum:		
Glucose	435	97
Mannitol	111	0
Arabinose	1138	398

* Oxygen consumption in culture without added carbon source taken as 100 per cent.

tion-reduction character of the medium. A mannitol-nitrate medium, for example, was found to be too highly oxidized for optimum growth of the organisms at the surface. The superiority of ammonium nitrogen over nitrate for utilization by *Rh. meliloti* might be attributed to the slightly reduced potential which should obtain in the ammonium media as compared with

that of the nitrate media. However, in the light of the results obtained with *Rh. japonicum* (table 3) it seems improbable that these phenomena can be attributed to the slight difference in potential induced by the addition of either form of nitrogen. The soybean organism showed a very much higher rate of oxygen consumption on nitrate media than on media containing ammonium nitrogen. It seems, therefore, to be merely a matter of the greater suitability of a particular form of nitrogen for utilization by one organism.

SUMMARY

1. No significant differences were observed in the rate and extent of oxygen consumption by *Rh. meliloti* in media containing glucose, mannitol, or sucrose. Galactose and arabinose were superior to glucose for utilization by this organism on both nitrate and ammonium media. Maltose, lactose, inositol, dulcitol, and sorbitol with both forms of nitrogen, and raffinose and erythritol with ammonium nitrogen were decidedly inferior to glucose as a source of energy. Ammonium nitrogen seemed more suitable for utilization by *Rh. meliloti* than nitrate nitrogen, as measured by the total consumption of oxygen.

2. Arabinose was distinctly superior to the other carbonaceous compounds studied, as a source of energy for *Rh. japonicum*. Glucose, galactose, and xylose permitted approximately equal amounts of oxygen consumption. Mannitol, mannose, maltose, glycerol, lactose, sucrose, and erythritol were utilized only slightly or not at all. Nitrate nitrogen seemed more suitable for utilization by *Rh. japonicum* than ammonium nitrogen.

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A STUDY OF ACID AND GAS FORMATION BY MEMBERS OF THE COLON-AEROGENES-INTERMEDIATE GROUPS IN THE PRESENCE OF CERTAIN SUGAR ALCOHOLS AND THEIR ANHYDRIDES

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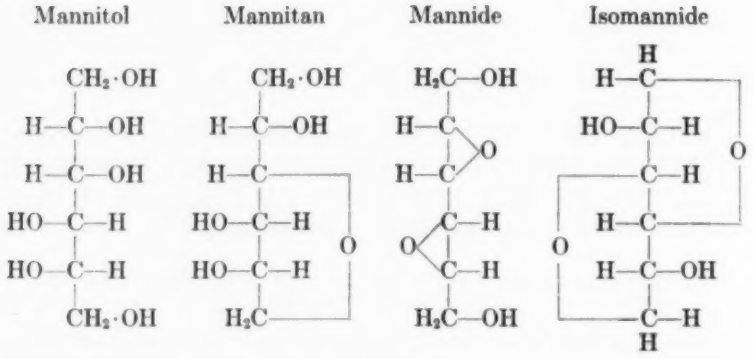
The use of dulcitol and mannitol as differentiating agents in bacterial culture media is well established. The mechanism of the decomposition of these compounds has been investigated by many workers. With mannitol, this field has been comprehensively reviewed by Eitel (1920). Our review of the literature, however, revealed no bacteriological investigations concerned with acid and gas formation from the anhydrides of these alcohols.

Our interest in the bacteriological examination originated in the work of two of us (Carr, Musser, Schmidt and Krantz, 1933; Carr and Krantz 1934) who investigated the fate of these sugar alcohols and their anhydrides in the animal body. So striking was the difference between the fate of mannitol and its first and second anhydrides, that it was considered of sufficient interest to extend these investigations to the field of bacteriology. In general, although the sugar alcohols are metabolized in the animal body (albino rat), their anhydrides resist metabolism and for the most part pass through the organism unchanged.

CHEMICAL CONSTITUTION

The following formulas show the structural relationships of the substances employed.



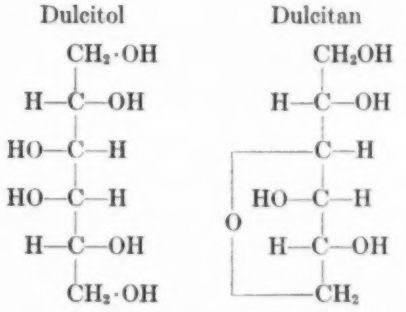
The mannitol used in this investigation was supplied through the courtesy of Merck and Company, Inc., Rahway, New Jersey. The aqueous solution of the compound (1:10) is neutral to litmus and optically inactive. The mannitan was prepared from mannitol by a slight modification of the method of Vignon (1874) in which dehydration is accomplished by means of sulphuric acid. Analysis gave the following composition: calculated, C 43.87, H 7.37; found, C 44.57, H 7.39. The compound was acetylated and the resulting ester distilled in vacuo. The fraction distilling between 200 to 210° at 10 mm. was saponified. The calculated percentage of CH_3COOH is 72.31, that found was 71.70.

The mannide was prepared from mannitol by the method of Liebermann (1884). Analysis gave the following composition: calculated, C 49.28, H 6.90; found, C 48.37, H 7.32.

The isomannide was prepared by the method employed by Fauconnier (1882) who used concentrated hydrochloric acid as a dehydrating agent. The isomannide, after purification was white, crystalline and melted sharply between 85 and 85.4°. The structure of isomannide was assigned through the researches of van Romburgh and van der Burg (1922).

Pfanstiehl's C. P. dulcitol, melting point 188°, free from galactose was used. The dulcitan was prepared from dulcitol by Berthelot's method (1860). Analysis gave the following composition: calculated, C 43.87, H 7.37; found, C 43.79, H 7.20.

The constitutional relationship is indicated by the following formulas.



METHOD OF STUDY

One hundred and twenty-seven strains of colon-aerogenes-intermediate groups were studied. These were distributed among the following species: *Escherichia coli*, *Escherichia communior*, *Escherichia neapolitana*, *Aerobacter aerogenes*, *Aerobacter oxytocum*, *Aerobacter cloacae*, *Aerobacter levans*. Certain strains, however, could not be grouped in either of these genera but showed characteristics intermediate between them.

Many of the microörganisms had recently been isolated from water, urine, feces, oysters, oyster water and necropsies, but a number of strains came from the Tittsler collection.

One per cent carbohydrate broth culture media were prepared by adding the sterile carbohydrates to sugar free nutrient broth.

The cultures, after inoculation, were incubated for one week and observations for the formation of acid and gas were made daily.

RESULTS

All of the species studied produced acid and gas from mannitol but none formed either acid or gas when grown in mannitan, mannide or isomannide broth. The cultures of *Aerobacter aerogenes*, *Aerobacter cloacae* and *Aerobacter levans* failed to produce acid or gas from dulcitol and dulcitan. Those of *Aerobacter*

oxytocum, *Escherichia coli* and *Escherichia communior* with the exception of five strains, fermented dulcitol with the formation of both acid and gas. These atypical strains conformed in all their other characteristics to *Escherichia communior*. The majority of the members of the intermediate group failed to attack dulcitol. None of the organisms studied was able to ferment dulcitan.

CONCLUSIONS

Previous work has shown that the anhydrides of dulcitol and mannitol, namely mannitan, mannide, isomannide and dulcitan are not metabolized in the animal body. Dulcitol and mannitol are fermented by many organisms of the coli-aerogenes group whereas their anhydrides are not utilized as determined by the ordinary fermentation tests.

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A MICROSCOPIC METHOD OF DISTINGUISHING DEAD FROM LIVING BACTERIAL CELLS

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For a long time the biologist has been attempting to discover a criterion by which a living cell can be distinguished from a dead cell by simple microscopic examination. The most popular method has been the application of dyes, either in the form of the so-called vital staining, or in the usual way on dried and fixed smears.

1. METHODS OF "VITAL" STAINING

Dilute solutions of dyes were applied to animal or plant cells nearly as far back as a century ago. By this means it was sought to gain an insight into various life processes of the cells, to demonstrate certain cellular structures, or to find out whether the cell were dead or alive. The work started by Ehrlich on nerve cells and blood cells, and the stimulus it gave other workers, have given us a respectable literature on the subject, to which contributions are still being made from time to time.

The first application of "vital" stains to bacteria was made by Metchnikoff (1887) in his immortal study of phagocytosis. He says:

> To convince myself of the bactericidal action of the microphages, I made use of an old solution of vesuvin which was not in condition to alter living bacteria but stained dead ones bright brown. By adding a few drops of this solution to preparations of leucocytic exudates, I was able to observe that the majority of the rods (of attenuated anthrax organism) inclosed in the protoplasm of the microphages immediately

took up the brown color, while the cells (microphages) remained colorless and continued to live, manifesting their amoeboid movements.

Among the basic anilin dyes, methylene blue and neutral red have been the most popular, especially neutral red, introduced and warmly recommended by Ehrlich (1894). Plato (1900) used that dye in his extensive investigations, and Metchnikoff (1901) himself adopted it in his later work.

Among "vital" staining methods may be mentioned that of Růžička (1905) who recommends the use of a staining solution made by mixing equal volumes of 0.05 per cent neutral red and 0.05 per cent methylene blue solutions in distilled water. A few drops of this mixture are spread over the surface of a clean slide and allowed to evaporate at 35°C. This leaves a thin, dry film of dye on which a drop of the bacterial suspension to be investigated is placed and examined under a cover-glass. Within a short time, living cells take on a violet color in which the red tone predominates. Dead cells show a predominance of blue tone.

Here belong also those "vital" staining methods with acid dyes, like that of Seiffert (1922) who used congo red. Seiffert's stain was a dilution of 1:10 of a saturated congo red solution in physiological salt solution. Others have used this dye in negative preparations. Dead bacteria are supposed to take up the dye, living bacteria to remain colorless.

2. METHODS OF APPLYING STAINS TO DRIED AND FIXED SMEARS

There is a variety of these methods described in the literature. The best known among these are Bordet's (1895) eosin-methylene blue method, and that of Proca (1909), especially in the form of Kayser's modification.

Bordet observed in the course of his studies of phagocytosis that phagocytosed bacteria gradually undergo a change in their staining reaction. At first they are cyanophilic and are colored blue, when the smear is stained with eosin and then with methylene blue. Gradually, under the action of the phagocyte they become eosinophilic and take up a pink color when stained in the same way. A continuous transition in color is seen between the two stages.

Proca's method consists in staining the dried and fixed smear for one minute with a solution made up by mixing 8 cc. of Ziehl's carbol fuchsin with 100 cc. of Loeffler's methylene blue and 100 cc. of distilled water. Dead bacteria stain red and living bacteria blue. Applied to spores (Proca and Danila, 1909), it was found that viable spores remain colorless and dead spores are stained blue. Proca also observed that bacteria killed by heat or disinfectants and stained with Loeffler's methylene blue, lose their blue color and turn red if subsequently stained with dilute Ziehl's carbol fuchsin (1:10). This observation was applied by Kayser (1912) in his modification. Kayser recommends staining of the smear three to five minutes with Loeffler's methylene blue, rinsing, and staining from five to ten seconds with Ziehl's carbol fuchsin, diluted 1:10. Recently this modification has been warmly recommended by Gay and Clark (1934).

The gram reaction has been suggested as a means of differentiating dead from living cells in gram positive species, and an improved method based on that reaction has been recently described by Frazier and Boyer (1934).

SIGNIFICANCE AND LIMITATIONS OF THE PRESENT METHODS OF DISTINGUISHING DEAD FROM LIVING BACTERIA

The above brief review gives the general lines along which methods, based on staining reactions, have been developed in an attempt to distinguish microscopically a dead from a living cell. In spite of some very optimistic reports, we may safely say that none yet has proved to be dependable. The following discussion shows why this is to be expected.

"Vital" staining methods

The basis of the use of vital staining has been so far indefinite. The general assumption is that living cells do not stain with "vital" dyes, while dead ones do. This assumption has never been satisfactorily justified, and investigators hold conflicting views on the subject. There is first the school led by Ehrlich which believed that the living protoplasm never stains, and that the appearance of stain is an evidence of death. Intracellular

structures which take up the stain are, according to this school, metabolic products taking no part in cell functions. In this connection we may mention the interesting "theory of micellar immunity" formulated by Lumière (1925), in which he explains the non-stainability of living protoplasm by its colloidal structure. The opposite camp is led by Przesmycki (1897), who believed that both the cytoplasm and the nucleus of the living cell may stain, and that, upon death, decoloration takes place. Similar views were held in recent years by Roskin and Semenoff (1933) and by Gavaudan (1933, 1934) and others. Between these two schools we find all kinds of transitional views expressed by investigators like Prowazek (1897), Arnold (1899) and Plato (1900). This latter investigator, although denying stainability of the living protoplasm in blood cells and in protozoa, yet believes that stained bacteria inside of the phagocytes may still be alive.

The above views regarding blood cells and protozoa were gained from microscopic observations of motility, or of the behavior of the stained intracellular granules. In one or two cases, claims of correlation with the division of a protozoan were made.

A different line of attack was followed by Fraser (1920) who compared the number of viable yeast cells as obtained by "vital" staining, with the number of colonies developing on the petri plate. After trying out a number of basic and acid dyes, Fraser adopted methylene blue. He found that the plate count was consistently less than the number of unstained cells and concluded that the cell loses the power of reproduction before it stains. Methylene blue was also used by Fulmer and Buchanan (1923). Rahn and Barnes (1933) used Congo red in addition to methylene blue and compared their results with the fermenting power and the plate count of yeast cells. Their conclusions are, likewise, that yeast cells lose the power of reproduction first, followed, in order, by the loss of fermenting power and by the acquisition of the ability to stain with the dyes used.

On going over the literature, one can but feel that much of what is assumed in using "vital" dyes to distinguish dead from living cells is arbitrary and indefinite. This feeling becomes even

stronger when one undertakes such a study experimentally. It seems natural to consider dead any cell that takes up a dye and living any one that does not; for, if we suspend growing cells in dilute dye solutions, they remain colorless; and, if we kill the cells by heat or chemicals, they immediately stain. But is every stained cell a dead one or every non-stained one alive? We have seen that the authorities are not in agreement on that point. And then we have the more complex question of degree of staining. How about cells that barely show color? And cells that contain stained vacuoles of various kinds? Much has to be determined before "vital" staining methods can become dependable.

Methods using dried and fixed smears

Even less is known of the principles behind the methods involving the staining of dried and fixed smears. In general, such methods are evolved empirically as follows: A smear made from a living culture and stained in a certain way shows certain color or predominance of a certain shade of color, while a culture, killed in certain ways and stained by the same method, shows a different color or predominance of a different shade. In attempting to explain these, at best, rough reactions, some wild assumptions have been made; for instance, the assumptions that in a dried film fixed by heat, the bacteria are still alive, if the film came from a living culture.

In order to get an idea of the value of these methods, the reader is referred to an article by Bickert (1930) who investigated a variety of them and arrived at the conclusion that none of them is of any practical value.

I, myself, have investigated the most recommended of all these methods, the Proca-Kayser method, and my conclusion is that it does not show the difference between dead and living cells, but merely the difference in the degree of destruction to which the cells have been subjected. In the case of very young cells of *Escherichia coli* killed by quick cooling from 45° to 5°C. (Sherman and Cameron, 1934) the method fails completely. There are two more objections to this method. First, the shade of

color is rarely pure blue or pure red. It is usually violet with a predominance of blue or red shades, and all kinds of transitional shades in between. Second, it is not suitable for quantitative work because many of the cells are washed off on rinsing.

PRESENT INVESTIGATIONS

A satisfactory solution of the present problem involves the solution of several preliminary problems. In the first place a suitable dye must be selected. After trying out several basic and acid dyes, neutral red was chosen on account of its low toxicity and its low reduction potential ($E^\circ = -0.330$ volts at pH 7). The concentration used was such that it had no retarding effect on the organism studied when added to the culture medium. In the case of *Escherichia coli* with which most of the present work was done, a concentration of 0.005 per cent was adopted, while some of the yeasts used required as little as 0.001 per cent of neutral red. It was also ascertained that, when the organisms were most actively growing in a neutral-red free medium, the addition of neutral red in the proper concentration caused no injury.

On the other hand neutral red has two disadvantages which, however, do not interfere with the method as it is here described. The first disadvantage is the fact that neutral red acts as an acid-base indicator in the pH range of 6.8 to 8.0. The alkaline color is yellow and hard to see under the microscope. The method automatically takes care of this in the process of diluting with neutral-red gelatin of pH about 6. The addition of a fermentable sugar to the original medium will also eliminate this trouble.

The second disadvantage is encountered when a strongly reducing organism like *Escherichia coli* is grown in a medium in which acid is produced. It has been known for a long time that *Escherichia coli* produces discoloration in neutral-red sugar media in the form of a yellowish-green fluorescence. Clark and Perkins (1932) have shown that this is due to an irreversible reduction of neutral red in the pH range of 4 to 6. Although the criteria and results given in this paper have been obtained by direct

experimentation and are independent of aberrations in the behavior of neutral red in the conditions described, yet numerous experiments were performed to elucidate the circumstances of the appearance of fluorescence in coli cultures. The conclusion from these experiments is that, when coli cultures in the neutral red medium used attain their most negative potential, the pH is about 6 and only about 25 per cent of the dye is reduced, which is not noticeable to the unaided eye. Fluorescence appears much later, usually in cultures twenty-four hours old or more, at 30°C. The pH of the culture is then about 5.2 and the dye should be reduced, only, to the extent of about 1 per cent. This apparently paradoxical fact has probably its explanation in the continuous accumulation of small amounts of the irreversible fluorescent material. As long as the bacterial population in fluorescent cultures is such that they must be diluted with neutral-red gelatin, the objection suggested by this phenomenon disappears.

The next step was to find out whether a cell can stain (in whole or in part) and still be alive. The test organisms were *Escherichia coli*, *Schizosaccharomyces pombe* and another yeast isolated from fermenting ale. Use was made of the technique of micro-cultures described by the author in a previous paper (Knaysi, 1933). The investigated organism was grown in glucose broth containing neutral red far below its inhibiting concentration. Micro-cultures were made from such cultures or from neutral-red glucose broth inoculated from these cultures. The micro-cultures were incubated and observed at room temperature. Each preparation contained about 30 droplets deposited in rows, and these were mapped immediately after the preparation was completed, and descriptions of the cells contained in some of them were recorded, and this correlated with viability. And, again, after growth in certain droplets had proceeded far enough or was completed, micro-transfers were made to fresh droplets of the neutral-red glucose medium or, in certain cases, the medium was sucked out and fresh medium added, thus insuring the observation of some particular cell or cells.

The conclusions drawn from numerous such experiments may be stated as follows:

1. Normal, healthy cells do not show the slightest evidence of color either of the cytoplasm or the vacuoles and other inclusions.

2. Any cell showing evidence of staining of its cytoplasm, no matter how faint it is, is a dead cell.

3. Cells in which the vacuoles *alone* are stained, may or may not be able to grow. In the first case, the vacuole loses its color completely before growth takes place. In the second, the stain diffuses out into the cytoplasm, giving it a faint tinge of color.

4. There is a certain number of healthy, normal-appearing cells that fail to grow, and yet they do not take up the dye for a long time. These were probably alive but somehow lost the power to reproduce. They are numerically few.

5. Before autolysis sets in, a dead cell stains deep red, but as autolysis proceeds, staining becomes gradually fainter, and one finds a certain number of cells that look disorganized and have a low refractive index without showing evidences of staining. Such cells are not numerous and are easy to recognize. They probably contain no more stainable material.

ADDITIONAL TESTS OF RELIABILITY AND SENSITIVITY OF THE METHOD

Aside from the direct viability test described above, other means have been used to gain an idea about how reliable and sensitive the method is.

Cultures of *Escherichia coli* killed by heating to 65°C., in flowing steam, or in the autoclave, take up the dye immediately, as do cultures killed by phenol and mercuric chloride. The dye may not penetrate the cell instantaneously on account of coagulation of the protoplasm or the like, but the membrane of the cell stains deeply and there will be no mistake about the cell being stained.

A most crucial test for the sensitivity of the method is to apply it to young cultures cooled quickly from 45° to 5°C. It may be remembered that Sherman and Cameron (1934) have recently noted that when very young, rapidly growing cultures of *Escherichia coli* are quickly cooled from 45° to 10°C., over 90

per cent of the cells may be killed. This discovery is surprising in view of the fact that both temperatures are included within the temperature range of growth of *Escherichia coli*. The criterion of death used by Sherman and Cameron having been the failure to grow on petri plates, it is reasonable to consider the possibility that cooling merely interfered with the growth mechanism of the cell instead of causing instantaneous killing. When the present method was applied to such cultures, it was found that almost all the cells took up the dye, indicating an immediate death.

The present method also shows that in a smear of *Escherichia coli*, dried and fixed by heat, the cells are dead, contrary to a statement of Ficker (1929) adopted by various investigators, including Bickert (1930) and Gay and Clark (1934).

In addition to the above tests it was also thought desirable to compare the numbers of viable bacteria as obtained by the present method with those obtained by the petri plate method.

NUMBERS OF VIABLE CELLS IN CULTURES OF ESCHERICHIA COLI, AS DETERMINED BY THE NEUTRAL RED AND BY THE PETRI PLATE METHODS

Technique

A certain volume of the culture of *Escherichia coli* in which it was intended to enumerate viable cells was diluted with 15 or 20 per cent of nutrient gelatin at 35°C., containing 0.005 per cent neutral red. The percentage of gelatin used depended on the degree of dilution and on the temperature of the room. The mixture was shaken thoroughly for a few minutes and replaced in the water bath until most of the foam disappeared. Then 1 cc. was measured into a 99 cc. water blank previously warmed to 30°C. and, immediately following this, a loopful of the bacterial suspension was used to fill a Petroff-Hauser counting chamber. The chamber was allowed to remain one minute at 37°C. while filling, in order to keep the viscosity of the gelatin low and prevent it from raising the cover-glass, which would introduce a large error. During the time the chamber was at 37°C., the water

blank which received 1 cc. of suspension was being shaken. The chamber was then placed in a 10°C. incubator to allow hardening of the gelatin and to slow down possible growth of the organism, and it was left there until plating was completed. This procedure, it was ascertained, causes no injury even to the most sensitive cells. The value of the gelatin is to check motility of the organism, and to supply rigidity, as counting was made with the oil-immersion objective.

The Petroff-Hauser counting chamber has similar ruling to ordinary haemacytometers and possesses the advantages of being adapted for use with the oil-immersion objective and with the dark-field condenser. Its depth is 0.02 mm.; that is, it is one-fifth as deep as the chambers of ordinary haemacytometers. An average of one cell per small square is equivalent to 20 million cells per cubic centimeter. With the possible large error involved, the degree of accuracy attained is remarkable, even when the suspension contained less than 10 million cells per cubic centimeter. Usually about 50 small squares were counted.

In plating, water blanks warmed to 30°C. were used. Warming the water blanks was for the purpose of keeping the gelatin in the liquid form. The medium used for plating was nutrient agar containing 0.5 per cent glucose. The agar was poured at 45°C. Before this procedure was decided upon, experiments were performed in substituting broth blanks for water blanks, and in using different percentages of agar to make pouring possible at 35°C. No advantage was found for any of these modifications, even with most sensitive cells. The glucose was incorporated, however, because it made the organisms grow much faster and form larger colonies. Counting was usually made after 24-hour incubation at 37°C.

Results

The results of comparative counts are recorded in tables 1 and 2. Table 1 includes counts of young cultures, while table 2 includes the results with mature and old cultures.

In comparing any microscopic count with the plate count, it is necessary to compare the number of groups observed under

the microscope with the number of colonies developing on the plate. By group, is meant one cell or two or more cells that are still united by their cell wall. This point seems too evident to need mention, and yet, strangely enough, in the few instances where a direct count of viable cells was compared with the plate count, no mention was made of groups, except in Frazier and Boyer's report. This, alone, is sufficient to magnify the discrepancy found between the two methods to the proportions recorded. The argument that, upon shaking, groups (in the sense here used) are broken up to individual cells does not hold in the

TABLE 1

Number of viable cells in young cultures of Escherichia coli as determined microscopically and by plating

The organism was grown in glucose broth containing 0.005 per cent of neutral red at 30°C.

EXPERIMENT	AGE OF CULTURE	MICROSCOPIC COUNT PER CUBIC CENTIMETER OF CULTURE			PLATE COUNT PER CUBIC CENTIMETER OF CULTURE	$\frac{\text{PLATE COUNT}}{\text{GROUP COUNT}} \times 100$
		Cells $\times 10^6$	Groups $\times 10^6$	$\frac{\text{Groups}}{\text{Cells}} \times 100$	Average $\times 10^6$	
	hours					
1	4	185.0	111.0	60.0	119.0	107.2
2	$4\frac{1}{2}$	107.0	60.0	56.0	67.3	112.0
3	$3\frac{1}{4}$	72.0	48.0	66.6	51.0	106.0
4	12	703.0	356.0	50.6	280.0	78.7
5	$1\frac{3}{4}$	2.2	1.1	50.0	1.4	127.2
6	$3\frac{1}{2}$	11.2	5.8	51.8	5.9	102.0

light of the figures reported in tables 1 and 2, even for as "individualistic" an organism as *Escherichia coli*. The gelatin suspension was usually vigorously shaken for nearly five minutes, and yet the ratio of groups to individuals never reached unity, although in a few experiments with old cells it approached that value. In young cultures of coli, where the cells are actively growing, the number of groups is nearly half of that of individuals, because most of the population consists then of pairs of sister cells. In cultures where growth is very slow, this circumstance is non-existent, and the cells tend to be single with few pairs and an occasional short chain.

When the number of viable groups is compared with the number of colonies, it is evident from the tables that these numbers check very closely. In the many experiments performed, there were some showing discrepancy. But the number of such experiments is small and almost reached the vanishing point as the

TABLE 2

Number of viable cells in mature and old cultures of Escherichia coli as determined microscopically and by plating

The organism was grown in glucose broth containing 0.005 per cent of neutral red at 30°C.

EXPERIMENT	AGE OF CULTURE	MICROSCOPIC COUNTS PER CUBIC CENTIMETER OF CULTURE			PLATE COUNT PER CUBIC CENTIMETER OF CULTURE			PLATE COUNT / GROUP COUNT ×100
		Cells ×10⁶	Groups ×10⁶	Groups / Cells ×100	Duplicate counts ×10⁶		Average ×10⁶	
	hours							
1	48	528.0	374.4	70.9	396.0	408.0	402.0	107.4
2	48	674.0	488.0	72.4	402.5	410.0	406.3	83.3
3	118	200.0	140.0	70.0	186.0	187.0	186.5	133.2
4	119	220.0	180.0	81.8	228.0	233.0	230.5	128.0
5	36	530.5	285.8	53.9	255.0	306.0	280.5	98.2
6	63	317.7	198.9	62.6	137.7	158.2	148.0	74.4
7	40	340.0	238.0	70.0	233.0	267.0	250.0	105.0
8	37	412.5	289.0	68.8	299.0	306.0	302.5	104.7
9	42	417.0	306.0	73.4	284.0	296.0	290.0	94.8
10	36	731.0	544.0	74.4	544.0	561.0	552.5	101.6
11	38	751.0	578.0	77.0	426.5	442.0	434.3	75.1
12	40	548.0	364.0	66.4	423.4	445.6	434.5	119.4
13	62	724.2	493.0	68.1	462.5	468.0	465.3	94.4
14	64	724.2	510.0	70.4	476.0	486.0	481.0	94.4
15	36	632.0	500.0	79.1	462.2	488.0	475.1	95.0
16	35	540.2	418.3	77.5	387.5	396.0 425.0	402.8	96.3

technique became more and more refined. On the other hand, in the great majority of the experiments, the agreement was surprisingly close, often closer than counts given by duplicate plates. The tables give a representative account of the results. Out of 22 experiments reported, 13 give an average number of 90 to 110 colonies, the group count being considered as 100. One

would have no objection if his duplicate plates came consistently as close as this. It must be added that, in most of the cases reported, the gelatin suspension contained less than 25 million per cubic centimeter, and in a couple of experiments with young cells it contained less than 2 millions. A much closer agreement is obtained when the number of cells counted in the gelatin suspension is made larger.

SUMMARY

This paper is concerned with the use of neutral red in distinguishing, microscopically, between dead and living cells.

The technique of micro-cultures was used to study the relation between the viability of a cell and its ability to stain in whole or in part. It was found that cells of *Escherichia coli*, *Schizosaccharomyces pombe* and a yeast isolated from ale are to be considered dead whenever the cytoplasm proper is tinged, even slightly, with stain. Yeast cells with stained vacuoles and unstained cytoplasm are weakened, sick cells that may or may not recover. In a culture, there is a certain number of cells that are not stained and look healthy, but are unable to multiply. These are numerically few. Others do not stain because their autolysis is too far gone. These look disorganized, have a low refractive index and are easy to recognize. Their number is usually not important.

When comparisons are made between numbers of viable cells in cultures of *Escherichia coli* as obtained microscopically, using neutral red as an indicator of viability, and numbers of viable cells as given by plating, a close agreement between the two is found when groups, instead of single cells, are taken into consideration.

The concentration of neutral red varies with the organisms. In the case of *Escherichia coli*, a concentration of 0.005 per cent was adopted. This concentration is harmless even to the youngest and most sensitive cells, and the organism grows readily in broth containing neutral red in that concentration.

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A QUANTITATIVE DIFFERENTIAL METHOD FOR COUNTING MIXED CULTURES OF BACTERIA

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Studies relating to the association of bacteria in mixtures have demonstrated the need of a practical technique for obtaining accurate growth curves of each of several species which are growing together.

The standard method of plate counting gave unreliable quantitative differential counts when used with mixtures of *Streptococcus faecalis* and *Salmonella Schottmuelleri*, *Escherichia coli* and *Salmonella schottmuelleri*, *Streptococcus faecalis* and *Escherichia coli*. In many cases the subsurface colonies were easily confused, one species with the other. In other cases, since subsurface colonies require twenty-four hours longer incubation than do surface colonies, diffusion of acid formed by one member of a pair rendered the differential medium useless. In all cases, however, the surface colonies were readily distinguishable, due to differences in size, coloration, or medium change. The method to be described is the result of an attempt to find a procedure suitable for mixed culture work that would give counts of an accuracy comparable to those obtained by using the standard plate counting method with pure cultures.

For this method Petri dishes (100 x 15 mm.) having porous covers are used. The media employed should be poured while quite warm, sufficient amounts being used to give fairly thick plates. The plates are allowed to harden and are placed top down, five or six deep, in a dry incubator for fourteen to eighteen hours prior to use.

The dilutions of the culture to be counted are made in the usual manner. The plates are removed from the incubator just

before using, a warm plate being important in bringing about rapid drying of the flooded plate. Of the dilution, 1 ml. is deposited on the agar surface near the edge of the plate. In delivering, the pipette is allowed to drain and then carefully touched to a dry portion of the plate's surface to insure uniform delivery.

The flooding of the plate is accomplished by removing the cover and tilting the plate back and forth in such a manner that the liquid floods its entire surface in a thin even film. Should excess liquid remain at any edge it may be flooded to the center by further tilting of the plate. With a little practice this flooding takes but a few seconds for each plate. After the liquid is evenly distributed, the cover is replaced and the plates left on a level

TABLE 1

Statistical analysis of counts obtained by the flooded and standard methods of counting bacteria

	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	F	S	F	S	F	S
Arithmetic mean (colonies)	35.40	35.67	44.14	45.37	80.69	79.74
Median (colonies)	35.00	36.00	45.00	45.00	81.00	79.00
Quartile deviation (±)	4.50	4.00	4.00	4.50	6.00	6.00
Standard deviation (±)	6.48	5.71	7.00	6.77	9.56	9.15
Coefficient of variation (per cent)	18.31	15.01	15.68	14.92	11.85	11.48
Number of plates	150	150	195	195	193	199

bench in a warm room, or placed top up in the incubator until dry. This is usually accomplished within twenty minutes. When dry the plates are incubated in the usual manner until sufficient colony growth has occurred. The distribution of colonies on the flooded plate is very uniform, surpassing that usually obtained in the standard plate.

To compare the accuracy of the flooded plate method with that of the standard plate method three correlation experiments with pure cultures, involving a total of 1082 plates, were made. In these experiments an equal number of plates were seeded by each method, the work being done in a manner insuring uniform conditions for both methods. The counts obtained were analysed statistically with the results shown in table 1.

From table 1 it is seen that although some of the measures indicate the standard method to be slightly more accurate, the difference between the two methods is statistically insignificant. Since the coefficient of variation is independent of both mean and number of samples taken this measure may be averaged for the total number of plates. These averages, flooded 15.34, standard 14.14, probably do not differ by a significant amount. The moving averages of the frequency distributions of the counts obtained by both methods give very similar curves which conform to those of normal distributions. From the results of these correlation experiments it is apparent that the flooded plate method gives counts of an accuracy equal to that of the standard method.

The flooded plate method has been successfully used in this laboratory in obtaining growth curves of both pure and mixed cultures and in the isolation of pure cultures from fecal samples.

PROCEEDINGS OF THE LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CONNECTICUT VALLEY BRANCH

SPRING MEETING, WESLEYAN UNIVERSITY, MIDDLETOWN, CONNECTICUT, MAY 11, 1935

MINIMUM TEMPERATURES FOR THE GROWTH AND FERMENTATION OF CERTAIN LACTIC ACID BACTERIA. *Milton J. Foter*, Department of Bacteriology, Connecticut State College, Storrs, Connecticut.

ACIDURIC MOUTH BACTERIA AND DENTAL CARIES. *T. G. Anderson*, Department of Bacteriology, Yale University, New Haven, Connecticut.

Sixty-five observations were conducted on saliva, tooth scrapings, and tooth decay material, using a modification of the quantitative procedure of Rodriguez. In 55, or 84.61 per cent, of the cases a high numerical incidence of acid-producing streptococci was noted. Lactobacilli were observed in appreciable numbers in 12, or 18.46 per cent, of the total. Tooth scrapings from two "caries-free" monkeys showed a high incidence of lactobacilli. Lysozyme could not be demonstrated in human saliva. Attempts to procure a bacteriophage against oral lactobacilli or streptococci were likewise unsuccessful. The implantation in the mouth of lactobacilli of intestinal origin was not accomplished. The introduced organisms were rapidly eliminated from the mouth.

So-called enamel decalcification was produced *in vitro* with oral streptococci and oral lactobacilli, and with the two types in symbiosis. As the result of cultural, biochemical and serological studies with strains of oral lactobacilli, oral streptococci and intestinal lactobacilli, each of these organisms is considered to be a distinct type. On the basis of high acid production and numerical incidence observed in this study, the oral streptococci should be regarded as a possible factor in dental decay. Other agencies are, without doubt, involved in caries production.

STUDIES IN ACID PRODUCTION BY ESCHERICHIA COLI AND AEROBACTER AEROGENES. *A. V. Syrocki and James E. Fuller*, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

The Clark and Lubs medium (as used for the methyl-red test) was modified by the use of 0.3 per cent instead of the usual 0.5 per cent buffer (K_2HPO_4). The medium was tubed in test tubes containing small amounts of iron filings. When *Aerobacter aerogenes* was inoculated into the medium and incubated for 24 to 48 hours the buffer in the medium was sufficient to neutralize the acid produced and consequently no iron was dissolved. With *Escherichia coli*, however, sufficient acid was produced to overcome the buffer action and to dissolve some of the iron. Dissolved iron was indicated by the potas-

sium ferricyanide test. Results can be had in 24 hours, and reactions are marked in 48 hours. Organisms employed were designated as *coli* or *aerogenes* on the basis of the methyl-red, Voges-Proskauer, sodium citrate, uric acid, and indol tests. With intermediate strains of the Escherichia-Aerogenes group the dissolved iron test agreed as well with the Voges-Proskauer and methyl-red tests as did either the sodium citrate or uric acid test.

A Comparison of Methods of Detecting Streptococci in Freshly Drawn Milk Samples. *W. N. Plastridge and F. J. Weirether*, Storrs Experiment Station, Storrs, Connecticut.

The relative efficacy of six methods used in detecting the presence of streptococci in samples of milk collected from those quarters of the udder of a cow affected with chronic streptococcal mastitis is expressed in per cent as follows: (1) Direct microscopic examination of whole milk 3.8, (2) direct microscopic examination of sediment 8.9, (3) blood agar plates streaked with whole milk 60.8, (4) blood agar plates inoculated by the dilution method 76.0, (5) blood agar plates streaked with sediment 77.2, and (6) microscopic examination of incubated milk 98.7. While the last-mentioned method was the most effective of those used, it revealed the presence of streptococci in 38 of 281 samples from apparently healthy quarters. Three of the 38 quarters yielded cultures which were identified as *Streptococcus mastitidis* (Group A of Plastridge et al.), and developed other evidence of mastitis within a period of one month. Cultures obtained from several of the remaining 35 quarters were identified as saprophytes. The significance of the finding of streptococci in incubated milk samples, in the absence of other evidence of mastitis, or during the first two and last four weeks of the lactation period, can be determined only by isolation and identification of the streptococcus found in the sample.

Further Studies on Immunization with Heterologous Antigen. *W. L. Kulp and A. G. Borden*, Department of Bacteriology, Connecticut State College, Storrs, Connecticut.

Mice were successfully immunized against Pneumococcus type I infection by vaccination with a living culture of *Alkaligenes viscosus*. The immunizing antigen was grown on blood agar, suspended in physiological saline and injected subcutaneously at five-day intervals until a total of eight doses was given.

Two and one-half times the lethal dose of Pneumococcus type I was injected into the immunized mice and into controls. All the immunized mice, and none of the controls survived.

Attempted immunization against this same pathogen, using living cultures of *Streptococcus lactis* met with limited success, only 20 per cent of the treated mice surviving the pneumococcus infection.

There may be an antigenic relationship between the capsular material of *Alkaligenes viscosus* and the Pneumococcus type I capsule. Work thus far has not proved this.

Further and more intensive research relating to this problem is in progress.

BACTERIAL VARIATION: AN INQUIRY INTO THE UNDERLYING PRINCIPLES GOVERNING THE CELL MORPHOLOGY OF BACILLUS MEGATHERIUM

LEO F. RETTGER AND HAZEL B. GILLESPIE

From the Department of Bacteriology, Yale University

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In a recent publication (1933) we discussed briefly the pronounced influence of environment upon cellular morphology in certain stock strains of *Bacillus megatherium*. The present investigation marks a continuation of our earlier study and attempts to throw light on the significance of the variant cell forms so frequently seen in *Bacillus megatherium* cultures, by inquiring into some of the more important environmental factors which govern their existence.

It is unfortunate that nearly all of the reports published in support of the theory of complex bacterial life cycles have rested largely upon evidence obtained from the study of non-living organisms in stained preparations. For many of us such studies cannot supply convincing evidence concerning the developmental or cyclic relations existing between cell variants. Furthermore, our observations on the very irregular distribution of variant cell types throughout a given colony cast additional suspicion upon any hypothesis that depends for its support on the examination of smears made at random from old cultures. In order to avoid fallacies that are inherent in the stained-smear method, we have worked during this entire investigation with living cells and have followed their formation and continued development, under the microscope. *B. megatherium*, because of its large size, lends itself admirably to this type of study.

MEDIUM AND METHODS

Plain nutrient agar containing 0.3 per cent Bacto-meat extract, 1 per cent Bacto-peptone and 1.8 per cent Bacto-agar, and

having a final reaction of pH 6.8 to 7.0, was employed as the basal medium throughout this investigation. It was sterilized in test tubes containing about 12 cc. each, and was clarified by high speed centrifugation immediately before use.

Our first attempts at microculture study were made on the tips of agar cones built up in deep-well depression slides, according to the method of Adolph and Bayne-Jones (1932). The hanging block device of Hill (1902), with certain modifications, was soon adopted, however, because it offered definite advantages when cultures were to be incubated and observed over long periods of time.

Preparation of hanging block cultures

Sterile nutrient agar (5 to 9 cc., according to the depth desired) was pipetted, after centrifugation, into Petri dishes and allowed to solidify. Blocks 1 cm. square were cut out of the solidified agar and after inoculation were placed on 22 x 50 mm. sterile coverslips, with their inoculated faces against the glass. They were fixed firmly in place on the coverglasses by running melted paraffin along their edges. The coverglass preparations were then inverted over rectangular moist chambers (6 x 22 x 50 mm.), and sealed to the supporting walls of the latter with paraffin. Chambers deeper than 6 mm. were not employed, because they would have necessitated the use of an unusually intense source of illumination. Undue exposure of cultures to the light was avoided. During use, the open end of the rectangular chambers was temporarily sealed with plasticine.

It was unnecessary and, in fact, inadvisable to introduce water into hanging block chambers except when they were opened to permit some aeration; at such times sufficient water was added to compensate for the moisture lost through evaporation. With proper care and manipulation microcultures could be kept under observation for several weeks.

Throughout these experiments the temperature of incubation was 30°C. This temperature was maintained during continuous microscopic observation by enclosing all but the barrels of the microscopes in specially built "incubator boxes." At other times

moist chamber cultures, or microscopes with microcultures in place, were held in an ordinary incubator, and removed to incubator boxes when temporary microscopic observations were made.

Bacterial cell outlines were traced with the aid of a Zeiss camera lucida, at a magnification of approximately 2100 diameters. A 20× compensating eye piece and a $\frac{1}{12}$-inch oil immersion objective were employed for drawing. In order to avoid overlooking important microscopic details, all bacterial cells were examined, after tracing, with 8×, 10× or 12× oculars.

Departures from the procedure described above were frequently made necessary by the nature of special problems. For example, early in our work we observed that cellular variation in *B. megatherium* was definitely influenced by associated growth, but that the massing of cells necessary to bring out variant types interfered with the optical conditions required for detailed microscopic examination. To obviate this difficulty, the following technique was employed.

A light inoculum was placed on the upper or coverglass surface of an agar block, its density being determined by the number of microcolonies desired. After the block had been sealed to the coverslip, and immediately before it was suspended over a micro-chamber, its free surface (lower surface in the chamber) was inoculated with the same organism by streaking over its entire area or any selected portion thereof. The underlying colonies thus provided served as associated growth during the experiment and exerted a very marked influence on the overlying test culture. In order to prevent the underlying culture from becoming so dense as to cut down markedly the light from the sub-stage lamp, a second sterile agar block was placed over the first, thus sandwiching in the underlying inoculum and causing it to develop in the form of a thin film, rather than as dense, thickly layered surface growth. This growth did not interfere with optical conditions, because it lay in a widely different optical plane.

At first, suspensions containing varying numbers of bacterial cells were placed upon the two surfaces of the primary hanging block. Colony localization and the relative positions of overly-

ing and underlying colonies were, with the use of this technique, matters of chance. We later found it possible, by employing moderately dry agar, to follow definite inoculation patterns, and thus to control to a very large extent the conditions required for satisfactory study. For example, the overlying growth was sometimes made to consist of a single colony at the center of the block, and the underlying growth distributed over one-half of the block's inferior surface, leaving the other half free; or, at other times, the underlying growth was made to develop as a relatively narrow ring beyond the border of the overlying colony. Inoculation designs could in this way be suited to the purposes of different types of experimentation (see figure 27, plate 1).

THE INFLUENCE OF SURROUNDING GROWTH ON CELLULAR VARIATION

Early in our investigation we observed that rather heavily inoculated microcultures rapidly developed variant cell forms (in from one to three days), and that such forms appeared first in the sections of colony peripheries which lay most nearly adjacent to other colonies. Variation in such cultures was quite extreme and gave rise to cells ranging in shape from long, branched and unbranched filaments, through tapering and clubbed rods to short cubical and globular cells occurring in chains. The predominating cell type in any section of a colony appeared to be directly related to the density of population around that point. On very lightly inoculated agar blocks, on the other hand, cellular variants appeared late in the history of the culture, were never striking in form, and developed in horizontal strata deep within the cell mass. The layer directly under the coverglass was composed of moderately long, uniform rods which quickly became granular and never showed any further development, but gradually autolyzed and disappeared. Their rapid death was probably due to the fact that they were cut off, by the cells lying between them and the agar block, from their supply of nutrients and oxygen. The layer nearest the agar, because it was able to continue its growth under unfavorable conditions for the longest time, showed the most complete development of coccoid

cells. Under such conditions peripheral cells developed normally for as long as eleven days.

In order to explain the observed outstanding differences in time of appearance and localization of variant cell forms on crowded, as compared to thinly seeded blocks, it is necessary to postulate some "variation-exciting" influence or group of influences associated with density of population. It seems reasonable to suppose that certain of the environmental changes which follow metabolic activity as, for example, altered H-ion concentration, oxidation-reduction potential (or free oxygen supply), decreased nutrients and increased metabolic products, may play a major rôle in stimulating cellular variation under the conditions of these experiments. Such variation-determining factors are, naturally, most concentrated at centers of population (colony centers) and gradually decrease in potency as they diffuse out through the hitherto unattacked medium. They become more pronounced as the cultures grow older and more luxuriant, and they accumulate more rapidly on crowded than on thinly populated blocks. Furthermore, they are particularly accentuated where the zones of influence of two or more colonies overlap. In high concentrations they appear to be definitely unfavorable to cell development. At somewhat lower concentrations, however, while still slowing down development, these factors seem actively to stimulate cellular variation. Cruickshank (1934) has offered a very interesting demonstration of the influence of such factors on bacterial luminescence.

On very thinly populated blocks these variation-stimulating influences accumulate slowly, and never become sufficiently concentrated at colony peripheries to influence cell morphology. At the centers of even well-isolated colonies, however, they do gradually increase in concentration and eventually cause the development of coccoid and globular forms. On densely populated blocks, on the other hand, they accumulate much more rapidly and growth at colony centers may be stopped so quickly as to prevent cell variation entirely. The peripheral cells on crowded blocks feel these unfavorable influences more slowly than do those within the colonies; but even there these environ-

mental changes soon become so marked as to produce striking variations in cell morphology, followed by gradual cessation of growth. Thus, cellular variation may be observed to occur in those parts of a culture where there exists a definite balance between factors that are unfavorable and those which are favorable to growth. Variation appears here to be the result of an attempt on the part of developing organisms to adapt themselves to unfavorable environmental conditions.

Because the cell crowding necessary to induce variant forms interfered to some extent with the optical conditions required for clear microscopic observation, the modified hanging block technique described above (page 212) was devised. The results obtained with this method agreed entirely with those just reported. If the inoculum on the inferior agar surface was large enough to result in solid and continuous growth over its entire area, no marked variation in the cells of the overlying culture took place. On four out of six culture blocks having heavy underlying growth, only delicate wisp-like colonies developed which were composed of "normal" rods. On the other two the lower growth was less dense and a few coccoid and wedge-shaped cells were seen in the overlying culture. No further changes took place, aside from granulation of the cells, because unfavorable factors accumulated so rapidly in the medium as to cause complete cessation of growth in the overlying culture before material changes in morphology had time to develop. The analogy existing between such cells and those located at the centers of colonies on thickly populated blocks (without underlying growth) is quite apparent.

With greatly decreased concentration and irregular distribution of the underlying growth, overlying cultures were able to continue their development for longer periods of time under *relatively* unfavorable environmental conditions. Striking variations in colony size and cellular morphology appeared in such microcultures. For example, during the first twelve to fourteen hours of incubation on a relatively crowded block the cells in the overlying culture developed normally, although many of them were somewhat shorter than those in well-isolated colonies of the same age. Later, numerous short, pointed or wedge-shaped

cells appeared at colony peripheries. By the sixtieth hour some microcolonies contained rather long, thin, sharply pointed cells in their borders, while other peripheries showed coccoid cells and shorter wedge-forms. Other colony edges revealed only normal rods.

After ninety hours many variant cell types developed in different sections of the block. Clubbed and pointed forms were quite common and ranged in size from extremely long thick cells down to cells so short that their length scarcely exceeded their thickness.

In one of our preparations, after one hundred and ten hours' incubation the probable function of the "runner-type" cell (see page 227) was clearly revealed. In several instances such cells carried growth out beyond their colony of origin to less densely populated areas, there to establish new colony units. The new microcolonies contained apparently normal cells, and the "runner" cells underwent autolysis after accomplishing their purpose. Their faint outlines could be seen for some time, however, stretching between mother and daughter colonies.

Occasionally a wide zone of very large long-handled clubs and long thick rods was seen developing beyond a ring of small wedge-shaped cells. Some of the clubbed cells had equally large swellings at both ends. Fringes of long, branched filaments later developed beyond the rings of clubbed cells. Long "rope-twists" or filaments wound around each other were also prominent at times. Autolysis, which was always observed in certain sections of aging cultures, became more and more pronounced, and was quite marked at the end of one hundred thirty-six hours.

Practically all of the cells having unusual morphology were hyaline and had the general appearance of being viable. Although they were often seen lying in masses of autolyzed cellular debris, they apparently resisted autolysis. Furthermore, they were too numerous to be the result of mere chance.

The relation between the morphology of the overlying cells and the concentration of the underlying growth was clearly apparent. The length and tapering tendency of wedge-shaped cells and the length of the "handles" of clubbed forms varied

inversely with the relative density of the lower (associated) growth.

In an attempt to control the distribution of the underlying growth and its relation to the overlying culture, six hanging blocks were set up. The overlying growth on each of them was made to consist of a single colony at the center of the block. One inoculated block, destined to serve as a control, received no underlying inoculum. Graded concentrations of a suspension of *B. megatherium* were streaked over one-half of the lower surface of each of the five remaining blocks, leaving the other half uninoculated. Figure 27, plate 1, shows the relative locations of overlying and underlying growths. During the entire period of observation (seventeen days), that portion of the overlying colonies which extended out over underlying growth (V) revealed essentially the same cellular changes as were observed in earlier experiments in colonies developing over heavy underlying growth; i.e., at the end of *fourteen hours* the peripheral cells in this section of the colony were already beginning to become globular. Later, clusters and fringes consisting largely of these globular forms developed. Such cells as were unable to effect an appreciable change from the original rod form became very granular and underwent autolysis. The peripheral cells on the opposite sides of the colonies, however, remained normal in appearance during the first five days. On the *sixth day* several long-handled clubs were seen developing at "X," a cleft formed where two parts of one of the colonies came together. By the *seventh day* many clubbed forms had appeared. They were rather generally distributed through the border cells, but seemed to be particularly numerous at "X," where they were first seen. On the *eighth day* many clubbed forms were still visible, but a fringe of long filaments had developed beyond them. Many pairs of these filaments had begun to twist themselves in rope-cord fashion. They continued to develop slowly from the *ninth* to the *seventeenth day*. Each rope or braid was made up of two twining filaments.

It was interesting to note that throughout these six experiments the different types of cellular variants appeared in rather definite sequence: first normal rods, then clubbed and pointed forms,

followed by thin long filaments, branched forms, and finally twisting filaments. This chronological appearance of variants, however, does not necessarily indicate the existence of a cyclic relationship between the various cell forms observed. One variant form was never seen to develop into another variant type; for example, although long branching filaments followed clubbed forms in their appearance in aging colonies, the former did not develop from the latter, but from "normal" rods which existed among the clubbed cells. "Normal" rods under varying environmental conditions developed into: (1) coccoid or globular cells; (2) wedge-shaped cells of different lengths; (3) clubbed cells of various lengths and (4) filaments of various lengths, twisted or straight, and with or without branching. Some of these variant cells were able to develop back into "normal" rods when they were transferred to a fresh medium. Their apparent cyclic occurrence was, we believe, in reality due to the progressive environmental changes which occurred in the medium on which they were developing, rather than to any inherent cyclic tendency in the species. Each type of cell came out in response to definite, delicately balanced environmental conditions.

THE INFLUENCE OF OXYGEN STARVATION ON THE CELLULAR MORPHOLOGY OF B. MEGATHERIUM

As a result of early indications that free oxygen may play a significant rôle in the development of variant cells, an attempt was made to study the effect of mechanical oxygen exclusion on cellular morphology. Hanging block cultures were prepared in the usual manner except that the lower surface of each block was entirely covered by a coverglass of sufficient size to project slightly beyond the edges of the agar. The space around the agar between the overlapping edges of this glass and the regular coverglass was filled with paraffin, thus completely sealing in the block. Minute amounts of atmospheric oxygen penetrated to the culture through the paraffin seal, but they were far too small to allow continued normal growth. Under such conditions colonies at first developed normally. Soon, however, as the oxygen supply approached depletion, due to its rapid utilization, the periph-

eral cells became shorter. After a further period, the length of which depended upon the number of colonies developing and the thickness of the agar block, all of the peripheral cells were coccoid or globular. In appearance the microcolonies resembled those which had developed over very heavy underlying growth. (Fig. 1, Plate 2).

Certain globular cells appeared to be less susceptible to the unfavorable influence of reduced oxygen supply than were others. They continued to develop slowly for some time after general growth had ceased, and formed clumps of globular bodies, some of which remained viable and unautolyzed for many days after all of the rod-shaped cells surrounding them had died and autolyzed. Such globular cells correspond, we believe, to the "round regenerative bodies" reported by Löhnis and Smith (1916 and 1923). The autolyzing cellular débris in which they appear closely resembles Löhnis' "symplasm." However, we can safely conclude that our globular cells developed, not from the so-called "symplasm," but from normal rods. The exact manner of their formation has been studied, and their reversion to normal rods has been repeatedly observed (see page 219). Although they were made to appear by the simple mechanical exclusion of oxygen, they resembled fully the globular forms reported above as arising under the influence of "associated" growth. (Figs. 3, 4 and 5, plate 2).

When free oxygen was re-admitted to sealed cultures by removing the lower coverglass, the relatively resistant globular cells quickly resumed development and their progeny soon reverted to long, hyaline rods. It appears that the globular cells in *B. megatherium* cultures may serve to carry the species through certain unfavorable conditions, thus supplementing the function performed by spores. This phenomenon may have special significance in so far as *B. megatherium* is concerned, since it is well known that its cells cannot produce spores under materially reduced oxygen tension (Bayne-Jones and Petrilli, 1933).

Resistant globular cells were invariably developed under conditions of reduced oxygen tension, providing the oxygen supply was not too suddenly or completely depleted. The number of

globular forms appearing in a culture was, in general, inversely proportional to the speed with which oxygen was exhausted. When the oxygen supply was cut off very rapidly and strict anaerobic conditions were maintained, growth ceased before cellular variation could take place, and all of the normal rods in the culture soon died.

Whether such globular cells, when formed, can live for an appreciable period of time in a definitely anaerobic environment has not yet been determined. It seems quite probable, however, that their viability under greatly reduced oxygen tension is at least much greater than that of "normal" rods.

RE-STIMULATION OF GROWTH WITH POTASSIUM PERMANGANATE

Since we had observed repeatedly that re-stimulation occurred at once when atmospheric oxygen was re-admitted to cultures the growth of which had been interrupted by its mechanical exclusion, and since mechanical exclusion of oxygen always resulted in the development of colonial and cellular types similar to those which were formed over heavy underlying growth, it seemed that re-stimulation should occur in overlying cultures if more oxygen could be supplied to them.

Oxygen could not be mechanically admitted to such cultures because it had been excluded biologically, through the oxygen demand of underlying cells. Hence, to impart a distinct oxidative character to hanging block cultures, various oxidizing agents were employed, namely potassium permanganate, hydrogen peroxide, potassium chlorate, sodium nitrate and methylene blue. These were applied to the lower surfaces of hanging block cultures in which growth had ceased due to crowding. Striking results have been obtained thus far only with potassium permanganate.

When a crystal of potassium permanganate was placed in a small drop of water on one corner of the lower surface of a hanging block culture in which growth had ceased, and was allowed to remain on the agar until the color of the permanganate had diffused out over one-fourth of the block, the growth of normal rods was stimulated where the medium was faintly colored and

for a short distance beyond that area. Colonies farther away from the $KMnO_4$ region did not change. Ninety-eight hours after the application of permanganate many long-handled clubs and very large, long tapering rods could be seen in areas just beyond the zones colored by the permanaganate. The parts of colonies that were just within the colored area contained "normal" rods.

A full explanation for the growth re-stimulating influence of $KMnO_4$ is not, as yet, at hand. Several factors may be involved, but it appears probable that the positive effects obtained depended on the ability of $KMnO_4$ to furnish available oxygen to the culture, since its action was like that of simple atmospheric oxygen when air was re-admitted to cultures from which it had been mechanically excluded. From the fact that normal rods developed in areas that were faintly colored by the permanganate, and that clubbed and tapering forms appeared immediately outside of this zone, in an area between that in which normal rod forms had been re-stimulated and that in which no renewed growth occurred, we were led to assume that a certain approach to oxygen starvation stimulated the development of tapering and clubbed cells. Of course, it is also possible that the permanganate in high concentration completely destroyed some variation-stimulating metabolic product or products which were only partly destroyed by lower concentrations. This phase of the investigation is being carried forward, and we hope that it may be possible, by regulating the amount of oxygen available, to control the type and degree of cellular variation.

LOCALIZATION OF VARIANT CELLS, AND ITS RELATION TO THE INTERPRETATION OF DATA OBTAINED FROM STAINED PREPARATIONS

During our investigation we found that morphological variants did not appear uniformly distributed in colonies, but occurred in definitely circumscribed areas. Three types of localization were observed: (1) horizontal stratification; (2) peripheral zone formation, and (3) clump or cluster formation.

The first type (horizontal stratification) was observed in well-

isolated colonies where, as we have pointed out, striking cell variants never appeared. In such colonies the centrally located cells gradually decreased in length, with repeated multiplication, some of them actually assuming globular form. Cells of varying length were definitely segregated into horizontal layers.

That stratification is not the result of microcultivation but occurs also in cultures aging under normal conditions, is indicated by the work of Legroux and Magrou (1920). Using histological procedures, they cut and stained cross sections of cholera colonies and demonstrated definite morphological and tinctorial strata. Kahn and Nonidez (1933), also using histological methods, found three sharply differentiated layers in old colonies of *Mycobacterium tuberculosis*. These layers varied in acid-fastness, the surface zone being made up almost entirely of non-acid-fast rods and granules, and the deepest layer of acid-fast cells. The authors believed that the surface zone was the youngest layer of the colony. It appears, however, from our experience with microcultures of *B. megatherium* that the opposite may be true.

The second type of localization (peripheral zone formation) occurred very frequently. The more extreme cell variants (branched, clubbed, pointed, etc.) nearly always developed, under the conditions of our experiments, in narrow fringes around the edges of colonies. This made microscopic observation and study of the development of such cells particularly easy.

Globular cells often showed the third type of localization. Under certain conditions (relatively slow oxygen removal) microcolonies were studded with many clusters of large coccoid cells. With more rapid oxygen removal fewer clumps of globular cells developed. Sometimes only one or two clusters appeared in a colony; the remainder of the cell mass slowly autolyzed. (Figs. 3, 4 and 5, plate 2).

Because morphological types are not uniformly distributed throughout aging colonies but, as we have shown, often occur at certain points in high concentration, ordinary microscopic slide mounts from such colones may fail to present reliable cross-sections of the colony population as a whole. Indeed, it is quite possible that, in making smears from an aging colony by the

customary procedure, one may at one time pick up only a purely amorphous mass of cellular debris, while at another time one's needle may happen to touch a portion of the culture which contains an appreciable number of cells of some definite morphological type. It seems clear that one should not assume from such an observation that the cellular forms seen in the second preparation developed from the amorphous material observed in the first; yet, much of the work supporting the theory of complex bacterial life cycles (including amorphous or symplastic stages) has rested entirely on the study of stained smears. Our attempts to observe in microcultures the development of cellular forms from amorphous matter have thus far given negative results. Wyckoff (1933 and 1934) reported similar failures with other organisms.

THE ORIGIN AND FURTHER DEVELOPMENT OF CERTAIN VARIANT CELL FORMS

Very few attempts have been made to study the progressive morphological changes which occur in undisturbed cells as they develop in an aging colony. Most investigators who have reported work with microcultures have stated that after a few hours' incubation these cultures became so crowded as to render them useless for further study. We have found, however, that such is not necessarily the case, and that observations on isolated individual cells can and should be supplemented by investigations on the morphology of cells living in developing colonies. Cells growing in an undisturbed colony are as nearly in their "common or customary" environment as it is possible for them to be while under observation. It seems that, by starting with a single isolated cell and following its progeny for many days under the microscope, we should be able to obtain a reasonably accurate picture of the life history of the species in question. This should be particularly true if microcultures are grown under varied environmental conditions. Certainly, such procedures are open to fewer criticisms than are the other methods which are available at the present time.

During the early rapid growth period it is, of course, impossible

to follow the development of any particular cell and its progeny for an appreciable time. This is not a serious drawback, however, since frequent observations have shown us that practically no morphological variation occurs in *B. megatherium* cultures during this period. Hill (1902) stated that branching may begin in a culture of *Corynebacterium diphtheriae* within five or six hours after inoculation. Gardner (1925), working with seven species, reported that early growth in some cases was rich in "Y" forms; and Wyckoff (1934) stated that young Mycobacterium cultures may show many branching cells. It is possible that some of these observations may be accounted for by the fact that branched organisms develop more slowly than do normal rods, and that when they are carried over with the original inoculum they may remain dormant on the block, and be visible for hours lying among the normal, developing rods.

Pleomorphic cells begin to form in microcolonies of *B. megatherium* long after the logarithmic growth period has past. They often develop at colony peripheries, because further back in the colony growth has already ceased. This tendency toward peripheral localization makes microscopic study of their formation and development particularly easy. Growth at this time is so slow that repeated camera-lucida drawings can be made.

We wish again to emphasize the importance of direct microscopic study of living cells and their development in the particular environment that called them forth. Lewis (1932), proceeding on this principle, but using a less specialized technique, gained a very interesting insight into the significance of certain variant cells of *Bacillus mycoides*. Ørskov (1927) tried to study the growth cycle of the pleuropneumonia organism by observing development at the edges of young cultures on a solid medium. These attempts, we believe, represent steps in the right direction. Only by studies of a similar nature can we hope eventually to unsnarl the mass of conflicting evidence bearing on bacterial pleomorphism and its significance.

Under certain relatively unfavorable cultural conditions (oxygen starvation, for example) growth and reproduction are both slowed down. But growth in size is apparently more sensitive

to these unfavorable influences than is cell division. When the rate of cell division is more rapid than the rate of growth in cell size, cells become progressively shorter as reproduction continues. This phenomenon, carried to its extreme, results in the formation of cells which are no longer than they are broad (see figures 1, 3, 4 and 5, plate 1). Cells which have reached this state of extreme shortness tend to round up and become globular, resulting in the coccoid and yeast-like forms which have so often been reported as occurring in the reproductive cycle of many bacillary species. Our data indicate, however, that they are not formed as the result of any unusual or specialized reproductive mechanism, but that they are the result of the repeated binary fission of rods whose growth in length has been greatly retarded. Wyckoff and Smithburn (1933), Wyckoff (1934) and Lewis (1932) have also reported this phenomenon in other species of bacteria. The globular bodies that develop in this way are more resistant to the increasingly unfavorable environmental conditions that cause them to develop than are "normal" rod forms. They can be seen in old colonies lying in masses of autolyzing cellular debris (figures 3, 4 and 5, plate 3), and resemble, we believe, the "round regenerative bodies" reported by Löhnis and Smith (1916, 1923).

We have been able to observe the "regeneration" of these round yeast-like cells under the microscope (see figures 6 through 17, plate 1). When placed on fresh agar, they return to "normal" rod forms by a reversal of the process which led to their formation. Growth in cell length occurs more rapidly than does cell division. After four or five divisions the culture is again made up of rods of normal length. Soon after globular cells are placed on fresh agar they begin to swell, but often they do not do so uniformly on all sides. At first one side of the cell stretches out more rapidly than the other, giving a curved, bean-shaped appearance to the organism. It is probable that the cell wall of the older globular forms becomes somewhat thickened or hardened. After one or two divisions, however, this difference is no longer apparent.

A second type of round cell was repeatedly observed in cultures of *Bacillus megatherium*. Similar forms have been reported by

other investigators as occurring in several species of bacteria, and have been referred to as buds, gemmules, conidia or gonidia.[1] In our microcultures these cells did not form by budding, but by fission (see figures 19 through 21, plate 1). The division occurred, however, not at the center of the cell, but well towards its distal end (see figures 20 and 26, plate 1). Under certain conditions such round end balls were seen to elongate slowly and to return to the normal rod form (see figures 21 through 25, plate 1). The rods which developed from round terminal cells were never seen to divide; the significance to the species of such cells is not yet known. They differ from the large globular yeast-like forms in that the end balls develop very early in the history of a colony, while the other globular cells appear much later.

In certain cultures we have observed an interesting cell type which grows very rapidly in length. It differs from the average filament in that it tapers gradually toward its distal end (see figure 18, plate 1). We have called it the "runner type" of cell, because it runs out from the colony of origin, outdistancing the cells which surround it. Its apparent function is that of carrying growth out beyond the unfavorable surroundings of the mother colony to fresh "soil." When the tip of such a cell gets well away from the mother colony it begins to divide, and a new colony develops from the cells arising from it.

DISCUSSION

The more or less indiscriminate use of the term "life cycle" has led to much confusion in the minds of bacteriologists. To some it signified changes in cell morphology which include pleomorphism and complicated reproductive processes, some of which (we believe) may not be essential to the permanent existence of the species. To others it implies morphological changes through which an organism must pass from any given stage back to the same stage as, for example, from the egg to the egg, or from the butterfly to the butterfly.

[1] The recent observations of Rudakov (1934) are of particular interest. He noted such globular forms in a member of the *Lactobacillus* genus and reported them as filterable.

Bacteria, in common with all of the higher forms of life, pass through a cyclic developmental process from generation to generation, however simple this may be as compared with the transformations through which many protozoal parasites go, to which the term "life cycle" must and does apply. The present controversy on variation and life cycle hinges, therefore, not on the existence of such a thing as a life cycle, but on the types or phases through which bacteria pass as a part of their life cycle. For example, does the passing of *Bacillus megatherium* from rod-to-rod-to-rod, with certain variation in cell size, constitute its life cycle? Or does the cycle include the sporulation phase, as we have long assumed? Or, finally, must we include under this term the various branched, clubbed, filamentous and coccoid cells which may be seen in cultures under certain conditions?

If cells of varied, and often of extreme, morphology represent real stages in the life cycle of the species in which they appear, and are not merely variant forms which arise in response to changing environment, it should be possible to show (1) that they possess full vigor, (2) that they develop from each other in more or less definite sequence, and (3) that they are essential for the maintenance of the species in full vigor.

We need not look far for variants among the higher forms of plant and animal life which have become such in response to certain environmental influences. They represent attempts at adaptation. The misshapen and bent-over pines on the sea coast or open arid plains which struggle for existence against strong trade winds, the dwarfed and scrubby oaks that grow on mountain sides above the tree line, and the long, scraggy yellow grass found underneath log or rock piles, are all examples of this type of variation. Although they constitute distinct departures from the normal, they cannot in any sense be regarded as phases of a life cycle. They represent last attempts of individuals to maintain themselves under unfavorable environmental conditions. There is, we believe, a striking analogy between such forms and the variant cells which we have observed in cultures of *Bacillus megatherium*.

SUMMARY

1. The cell morphology of *Bacillus megatherium* is governed largely by environmental conditions. Relatively slight changes in environment are responsible for striking changes in cell form.

2. Cellular variation can be related directly to the degree of growth crowding and, consequently, to changes which take place in the medium as a result of increasing metabolic activity.

3. The factors which stimulate cellular variation are apparently unfavorable to continued normal growth. When they accumulate very rapidly in a culture development ceases before variation can take place. In other words, variation is possible only when favorable and unfavorable influences are so balanced as to permit slow growth in the face of untoward circumstances.

4. Partial oxygen starvation has been shown by us to be directly responsible for at least certain types of cell variation.

5. It appears that some variant cells are more able to resist the unfavorable conditions that call them forth than are the "normal" rods, and hence may be regarded as adaptation forms which aid in the preservation of the species.

6. We have had no evidence as yet which would indicate that definite cellular forms develop from finely granular or apparently structureless material. We wish to stress again the importance of subjecting cultural material to rigid and continued microscopic examination in life cycle studies.

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PLATE 1

With the exception of figure 27, the illustrations in this plate represent camera lucida drawings of living cells of *B. megatherium* developing on extract agar hanging blocks. ×1000.

FIG. 1. Chain of globoid cells from hanging block culture which had been held under partial anaerobic conditions for nineteen days.

FIG. 2. Embryonic cells of *B. megatherium* forty-five minutes old on hanging block.

FIG. 3. Periphery of nineteen hour old culture on agar cone. Relatively heavy inoculum and limited oxygen supply.

FIG. 4. Same cells four hours later (age twenty-three hours).

FIG. 5. Same cells nine and a half hours later (age twenty-eight and a half hours).

FIG. 6. Chain of globoid cells in hanging block culture which had been held under partial anaerobic conditions.

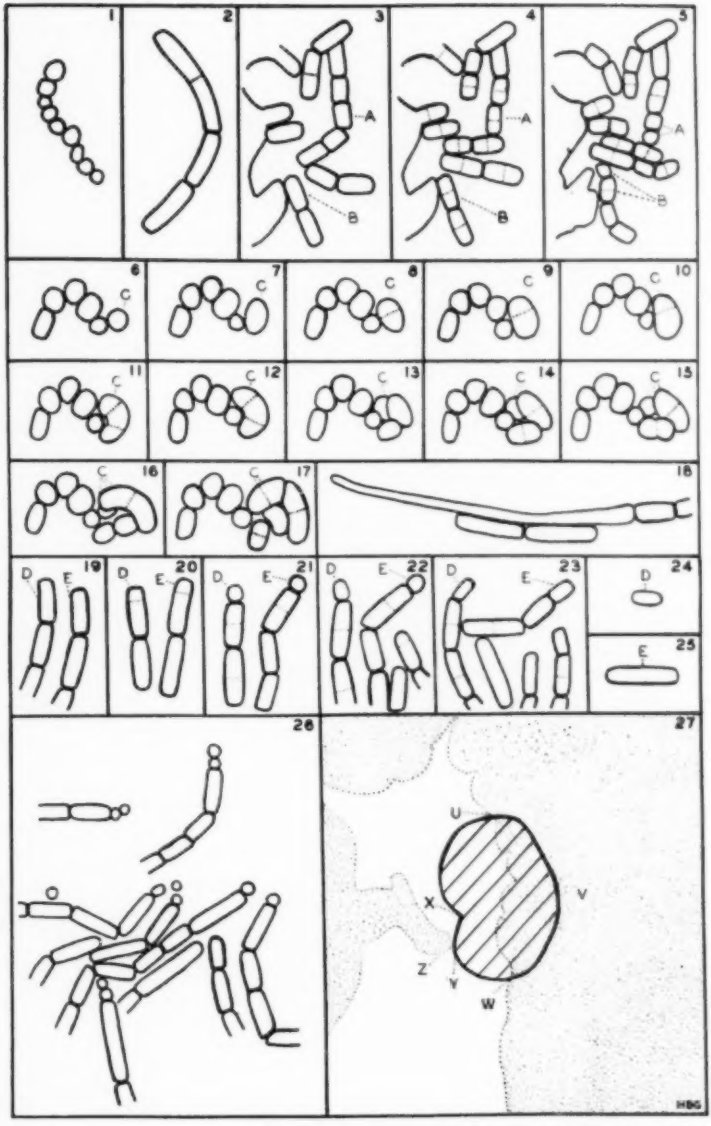
FIGS. 7–17. Same after lower coverglass had been removed to admit air. Drawings made at approximately thirty-minute intervals.

FIG. 18. Runner type cell at periphery of culture three and a half hours old.

FIGS. 19–25. The formation and further development of "round end balls."

FIG. 26. Periphery of twenty and a quarter hour culture showing "round end balls."

FIG. 27. Diagrammatic representation of overlying colony (diagonally lined) and underlying growth (stippled) on a square centimeter hanging block of agar.



(Leo F. Rettger and Hazel B. Gillespie: Bacterial variation)

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PLATE 2

Microphotographs of cells growing on hanging blocks of agar

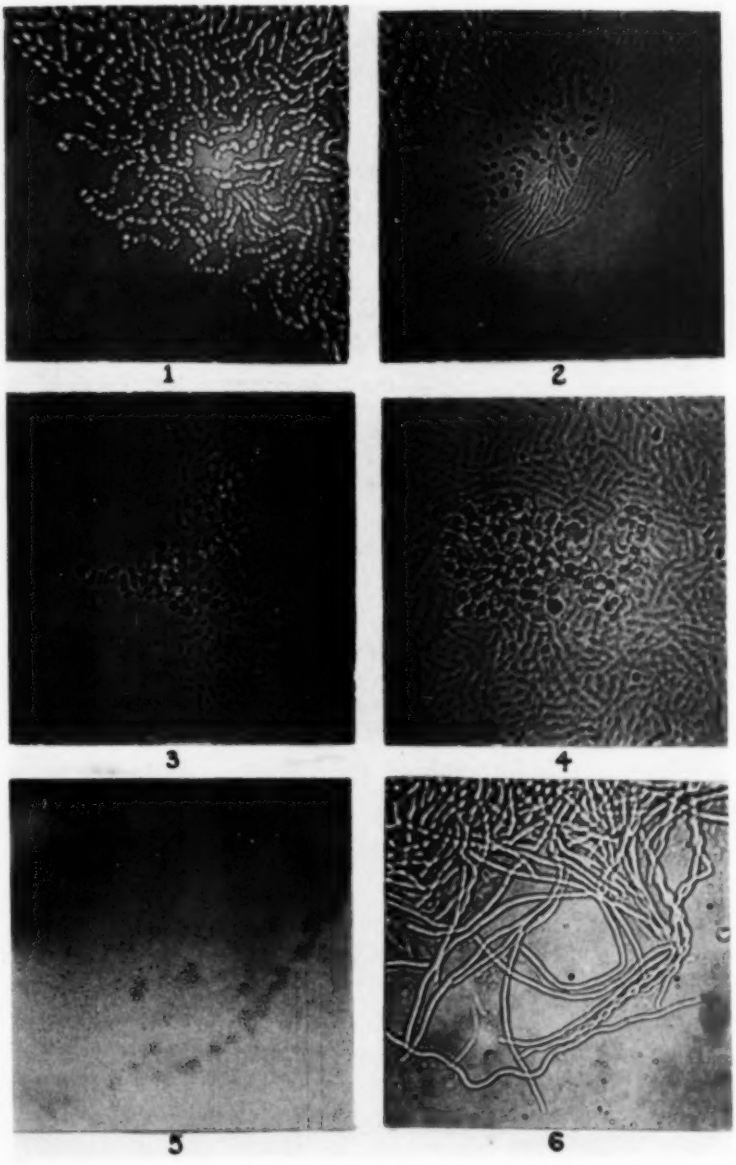
FIG. 1. Globular cells at the periphery of a colony developing under conditions of partial oxygen starvation. ×1000.

FIG. 2. Globular cells developing in a mass of dead and granular "normal" rod forms. ×1000.

FIGS. 3 and 4. Clusters of globular cells lying in masses of dead and autolyzing rods. ×1000.

FIG. 5. Periphery of colony similar to that in figures 3 and 4; shows further autolysis and gives appearance resembling Löhnis' symplasm with round regenerative bodies. ×80.

FIG. 6. Twisting filaments at periphery of a colony. ×1000.



(Leo F. Rettger and Hazel B. Gillespie: Bacterial variation)

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DIFFERENCES IN EFFECT OF PHENYL MERCURIC CHLORIDE UPON DIFFERENT RACES OF BACTERIOPHAGE AND SIMILARITY IN EFFECT UPON A PHAGE AND ITS HOMOLOGOUS ORGANISM[1]

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It has long been recognized that antiseptics and certain physical agents produce a deleterious effect upon the lysing ability of bacteriophage. Thus Eliava and Pozerski (1921) found that Shiga bacteriophage was destroyed by standing in a medium with a pH of below 2.5 or above 8.4. D'Herelle (1926) found that staphylococcus bacteriophage was markedly attenuated in fifteen days, and destroyed in thirty days, by 1 per cent potassium cyanide. He also found that acetone, glycerol, chloroform, various essences, and phenol destroyed it in varying periods of time. Other workers have found deleterious effects from lactic or oxalic acid (de Poorter and Maisin, 1921), antiformin (Seiffert, quoted by d'Herelle), trypaflavine (Wolff and Janzen, 1922), formaldehyde and acriflavine (Frisbee and MacNeal, 1930).

D'Herelle concluded that the effect of an antiseptic depends upon the virulence of the specific race attacked; this closely parallels his impressions concerning the effect of heat.

Few, however, have noticed a variation in the effect of one antiseptic upon different races of phage. Watanabe (1923) did observe a variation in the resistance of phage to alcohol, some races being destroyed in two hours, others requiring twenty-four. He also found that certain races could be destroyed by 1 per cent phenol, while others required 4 per cent. That there is a time factor, d'Herelle indicated after studying the effect of acetone,

[1] Prepared with the aid of a grant from the Isaac Kaufmann Foundation.

staphylococcus phage being destroyed in fifteen days, Shiga in twenty-five.

That temperature is also a factor was indicated by Bronfenbrenner and Korb (1924) who found that bacteriophage was rapidly destroyed by absolute alcohol at ordinary temperatures, but resisted for five or six days if held at 0°C.

Concerning the mercurial antiseptics more in detail, d'Herelle (1926) found that a 0.5 per cent solution of mercuric chloride did not affect phage suspensions in normal saline in three days, but did in four. Jamieson and Powell (1931) stated that bacteriophage was not markedly affected by merthiolate in 1:5,000 dilution during a course of a year. This finding was not corroborated by Straub and Applebaum (1933) or by ourselves in some unpublished studies.

It was determined to find the effect of a certain antiseptic—phenyl mercuric chloride (furnished through the courtesy of Prof. E. E. Ecker of Western Reserve University) upon different races of bacteriophage. A peculiarity of this extremely potent antiseptic, is its much greater effectiveness toward staphylococci than toward *Escherichia coli*.[2] Thus, for the organisms used in our own experiments, growth of the staphylococci was inhibited by a concentration of phenyl mercuric chloride of 1:2,000,000 while *Escherichia coli* could only be inhibited in dilutions no greater than 1:160,000. Another peculiarity of this antiseptic is that its presence does not inhibit the digestive action of trypsin and pepsin, or the lytic action of egg-white lysozyme (Ecker and Weed, 1933).

The titre of the bacteriophages used was at least 1×10^{-8} against the specific organism by the serial dilution method. Savita medium with a pH of 7.6 was employed throughout.

A protocol of the experiment is herewith presented:

Tubes containing 4.8 cc. of Savita medium had added to them respectively: nothing; 0.1 cc. of a specific bacteriophage; 0.1 cc. of the specific bacteriophage mixed with phenyl mercuric chloride so as to make a dilution of 1:20,000; 0.1 cc. of phenyl mercuric chloride diluted

[2] See Council on Pharmacy and Chemistry, 1934. Also confirmed by personal communication from Prof. E. E. Ecker of Western Reserve University.

1:20,000 in the medium; and similar tubes containing phage mixtures with phenyl mercuric chloride and phenyl mercuric chloride alone in dilutions of 1:40,000 and 1:80,000. The phenyl mercuric chloride was in contact with the bacteriophage for thirty minutes before being added to the 4.8 cc. quantities.

CHART A

Model of tube series to study effect of phenyl mercuric chloride on bacteriophage

TUBE LETTER	TUBE CONTENTS
A	Bacteria (0.1 cc. 12-hour culture). Used as bacterial control and source of next generation
B	Bacteria plus specific phage (0.1 cc.). Used as phage control
C	Bacteria plus mixture phage—PMCl 1:20,000 (0.1 cc. added)
D	Bacteria plus mixture medium—PMCl 1:20,000 (0.1 cc. added)
E	Bacteria plus mixture phage—PMCl 1:40,000 (0.1 cc. added)
F	Bacteria plus mixture medium—PMCl 1:40,000 (0.1 cc. added)
G	Bacteria plus mixture phage—PMCl 1:80,000 (0.1 cc. added)
H	Bacteria plus mixture medium—PMCl 1:80,000 (0.1 cc. added)

Typical readings from experiment conducted with staphylococcus

(All tests run in duplicate. Twelve hours incubation, 37.5°C.)

TUBE	GENERATION				
	I	II	III	IV	V
A (bact. control)	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
B (phage control)	Lysed	Lysed	Lysed	Lysed	Lysed
C	Lysed	Lysed	Lysed	Lysed	Lysed
D	Original turbidity	Original turbidity	Cloudy, beyond PMCl inhibition limits		
E	Lysed	Lysed	Lysed	Lysed	Lysed
F	Original turbidity	Original turbidity	Cloudy, beyond PMCl inhibition limits		
G	Lysed	Lysed	Lysed	Lysed	Lysed
H	Original turbidity	Original turbidity	Cloudy, beyond PMCl inhibition limits		

To these tubes, 0.1 cc. of an actively growing, twelve-hour culture of *Staphylococcus aureus* from a case of chronic furunculosis for which the phage was specific, was added. This was enough to produce a definite cloudiness, sufficient to distinguish it from an unplanted or lysed tube.

Duplicate series were made and the tubes incubated for twelve hours at 37.5°C. (for typical readings, see chart A).

A loopful (1 mm. loop) was then taken from each of the tubes showing lysis and those containing phenyl mercuric chloride and was inoculated into a new series of tubes. To these, 0.1 cc. of the previous, pure bacterial control was added. These tubes, plus a control, were now incubated for twelve hours at 37.5°C.

The process was then repeated, loopfuls from the lysed tubes and those still showing the inhibitory action of phenyl mercuric chloride being transferred, with twelve-hour incubation at 37.5°C. following each transplant.

Similar steps were carried through the fifth generation, all evidence of phenyl mercuric chloride inhibition disappearing after the third transplant, those tubes becoming as cloudy as the pure bacterial controls.

It was found that the lytic principle persisted equally well in the tubes to which bacteriophage alone had been added as in those to which phage mixed with varying dilutions of phenyl mercuric chloride was present. This lysis occurred far in excess of the dilution limits within which phenyl mercuric chloride inhibited bacterial growth.

A strain of *Escherichia coli* from a case of pyelitis and its specific bacteriophage was then substituted for the staphylococcus, and the experiment conducted exactly as before. Similar results obtained, except that the inhibitory action of phenyl mercuric chloride was evident only in the first generation.

It was then determined to find what action phenyl mercuric chloride might have when in contact with the bacteriophage for a period longer than thirty minutes. The tubes containing the original mixtures of phenyl mercuric chloride and the specific bacteriophages were stored at room temperature (approximately 25°C.) for six weeks. They were then planted as in the first experiment, two duplicately controlled series, one of staphylococci and one of *Escherichia coli*.

This time, the results were different. As before, there appeared to be no deleterious action by the phenyl mercuric chloride upon *Escherichia coli* bacteriophage. The lytic principle persisted through five transplants.

The staphylococcus bacteriophage, however, appeared to be

completely destroyed. There was no evidence either in liquid media or by plaque formation on solid media of any lytic activity. Attempts to recover the phage by three serial filtrations were all unsuccessful.

COMMENT

The variations in the effect of phenyl mercuric chloride on two different races of bacteriophage arouse speculations about its still undetermined nature. The fact that phenyl mercuric chloride does not destroy certain enzymes (Ecker and Weed, 1933) implies that, at least, coli phage could be an enzyme as advocated by Bronfenbrenner (1928) and others. On the other hand, the toxic action of the antiseptic upon staphylococcus bacteriophage and the homologous organism and the lessened toxicity upon coli phage and its organism give rise to the possibility of a linkage between the bacteriophage particle and the specific bacterium. The observed facts are not inconsistent with Hadley's conception of bacteriophage as a stage in the life cycle of the bacterium (1928).

SUMMARY

It was found that:

1. Phenyl mercuric chloride in dilutions of 1:20,000, 1:40,000, and 1:80,000 had no effect upon the lysing ability of either staphylococcus or *Escherichia coli* bacteriophages when in contact for thirty minutes.

2. When in contact for six weeks, however, at room temperature, the lysing ability of the staphylococcus phage was completely destroyed, while that of the colon bacillus remained as effective as the untreated controls.

3. The toxicity of the antiseptic upon the specific bacteriophage appeared to parallel that upon the homologous organism.

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STUDIES UPON GROWTH PHASES OF CLOSTRIDIUM SEPTICUM

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It is becoming increasingly evident that many bacteria may exhibit extreme morphological changes, even during their stages of rapid growth. *Clostridium septicum* (*Vibrion septique*) has been recognized as an organism of this type from the time that it was originally described by Pasteur and Joubert in 1877. When grown in an ordinary culture medium, such as chopped-veal infusion broth, it remains a small, somewhat irregularly staining Gram-positive rod which readily forms spores. An entirely different picture presents itself, however, when this bacterium is injected intramuscularly into a guinea pig. The animal usually dies within twenty-four hours and a smear from the local lesion shows the presence of numerous Gram-positive rods which vary considerably in size and shape; furthermore long Gram-positive filaments are visible when a smear is made from the surface of the liver.

It has recently been our experience that several members of the genus *Clostridium* will grow extremely rapidly in the body of a mouse if the animal is injected intravenously with these organisms, killed shortly thereafter, and incubated for a few hours at 37°C. A smear from the liver of a mouse, that had been injected in the above manner with *Cl. septicum* six hours previously, showed the presence of long Gram-positive filaments. It was decided to attempt to follow the morphologic variations of this organism in greater detail.

A pure culture[1] of *Cl. septicum* was transferred through chopped-veal infusion broth for three successive days, the final eighteen-hour culture being employed in the actual experiment. At the beginning of the experiment this culture was injected intravenously in 0.5 cc. amounts into 24 mice, which were then killed within three minutes after injection and incubated at 37°C. It was also inoculated in 0.1 cc. amounts into 15 tubes of chopped-veal infusion broth, which had previously been boiled for ten minutes and then cooled. One-third of these cultures was placed in each of 3 "McIntosh-Fildes" anaerobic jars which were quickly closed and incubated. Two mice were autopsied every two hours and smears were made from the livers and heart interiors. A jar was opened at these times, a smear was made from one of the cultures, and the jar was closed immediately. Inasmuch as three jars were employed it was only necessary to open each one every six hours, thereby causing a minimum disturbance of the anaerobic conditions under which the cultures were growing. All smears were subsequently fixed with methyl alcohol and stained by Gram's method.

The following gross findings were noted in the mice at autopsy: The animals autopsied at two, four and six hours appeared normal. At eight hours the tissues were slightly reddened and an excess of colorless peritoneal fluid was found. No gas formation was seen. The serous membranes were smooth and glistening. The mice autopsied at ten hours showed a moderate amount of gas in their peritoneal cavities, which also contained small quantities of a bright-red serous exudate. The livers and hearts were bright red, smooth and glistening. Gross blood was observed on section of the hearts. At twelve hours a bright-red gaseous

[1] This strain of *Cl. septicum* was obtained from the University of Colorado through the courtesy of Dr. Ivan C. Hall. On injection into the thigh of a guinea pig it produced a typical "cherry red," spreading gaseous edema and caused death within twenty-four hours. On autopsy it was found in the form of long Gram-positive filaments upon the liver surface, and it was recovered in pure culture from the heart's blood as a small motile Gram-positive rod. This culture, when streaked on a rabbit blood agar plate and incubated anaerobically, produced a delicate spreading growth which caused a slight amount of hemolysis. It fermented glucose, maltose, lactose and salicin with the formation of acid and gas, and had no effect upon mannitol and sucrose.

edema was present in the axillae and over the throats. The peritoneal cavities contained gas and a thin bright-red exudate. The liver surfaces were dull and roughened and these organs were somewhat friable. The hearts still contained gross blood on section. At fourteen hours the amount of gas throughout the bodies had become more noticeable. The livers and hearts were shrunken and friable, their surfaces were dull, and no gross blood could be seen when the hearts were opened. In the mice autopsied after fourteen hours the amount of gas formation and the drying of the tissues increased progressively.

A study of the smears made from the mouse livers and heart interiors at intervals following injection of the eighteen-hour *Cl. septicum* culture, yielded the following results: Smears made at two- and four-hour intervals showed little evidence of bacterial multiplication. Relatively few organisms could be seen, but some of those observed were moderately swollen when compared with organisms smeared from an eighteen-hour chopped-veal infusion broth culture of *Cl. septicum* (fig. 9). The six-hour picture was very different, presenting many uniformly swollen filaments, which varied in length from a few microns to long structures that often extended completely across the high power field. Some of these filaments were intensely Gram-positive, others exhibited more irregularity in their Gram-staining properties. Some filaments were of a very uniform structure (fig. 1), while others showed beginning division into a number of shorter segments (fig. 2). In addition to the filaments many short, uniformly swollen, Gram-positive rods were present. These forms appeared to have been taken from a rapid phase of growth and division.

Smears made at the end of eight hours still showed many filaments. At this stage, however, the filaments were less swollen, they demonstrated a greater tendency toward breaking up into short segments (fig. 3), and their Gram-staining reactions were increasingly irregular. At the ten- and twelve-hour intervals the filaments had almost completely broken into shorter segments, most of which did not exceed 15 microns in length. These segments varied considerably in their Gram-staining re-

actions and in the amount of swelling exhibited. In addition to this, many of them contained two or more very intensively staining large Gram-positive granules, whereas the remaining portions of these organisms took the stain much more lightly (fig. 5). After fourteen hours these granules were even more in evidence. At this time spores began to appear. These spores could be seen in all stages of development and seemed to be formed by changes in some of the above granules. The change from a solid-staining granule to a spore apparently involved a swelling of the granule followed by a clearing of its central portion,—the end result being a typical refractile spore with a deeply staining circumference (figs. 6 and 7).

From this point onward the picture became more confusing. Many different forms appeared. After a spore had formed in a bacterium, the organism appeared to shrink, the end result of which would seem to be a free spore. Many short forms were present, some Gram-positive, others Gram-negative. Some possessed granules, while others did not. A rather typical form at this stage was a short organism greatly swollen at the center with a granule at each end (fig. 8). A few small filaments could also be seen. This picture persisted up to the end of twenty-four hours, becoming increasingly complicated toward the end of this time by the fact that other organisms were apparently beginning to multiply within the tissues.

CULTURE EXPERIMENTS

As noted above a series of cultures were made in chopped-veal infusion broth at the time that the mice were injected. These cultures were incubated under anaerobic conditions and one of them was opened, smeared and stained at the end of each two-hour interval. These slides did not exhibit the extreme morphological changes shown by the organisms taken from the animal bodies. The bacteria from the cultures, were, in general, much smaller than those from the mice. However, some filament formation and some development of granular forms with subsequent spore formation from the granules was noted. It was

decided to try to duplicate the conditions within the animal body more faithfully in the culture tube.

A series of chopped-veal infusion broth tubes, containing one tenth of their volume of sterile human serum were made up. These tubes were kept in an anaerobic jar for a week at room temperature. They were then removed from the jar, tested for sterility, and each tube was inoculated with 0.1 cc. of an eighteen-hour chopped-veal infusion broth culture of *Cl. septicum*. They were then quickly placed in four anaerobic jars which were incubated at 37°C. One jar was temporarily opened every two hours for twenty-four hours and smears were made from a culture at that time as in the previous experiment.

On this occasion changes were observed which closely simulated those shown following mouse injection. Many long and swollen filaments (fig. 4) were seen and all stages of filament division, development of granules and spore formation were noted. During the early stages of this experiment the bacteria were generally very large when compared with those taken from plain chopped-veal infusion broth cultures of the corresponding period and approached in size the organisms smeared from the corresponding mice. In the last few hours of the experiment, however, they had become considerably smaller and appeared very much like any ordinary eighteen- to twenty-four-hour chopped-veal infusion broth culture of *Cl. septicum*.

DISCUSSION

The above experiments appear to us as evidence of the extreme variation of form and staining reaction that may be shown by a certain organism under suitable growth conditions. The question might be raised as to whether or not the various forms observed in the mice could be explained by the growth of extraneous organisms already present in the animal bodies. We have, however, on other occasions passed *Clostridium Welchii*, *Cl. sporogenes*, *Cl. oedematiens*, *Cl. histolyticum* and *Cl. Sordellii* through mice for ten hours in a similar manner, and in none of these instances were any forms observed that could be compared at all with the extreme examples of morphological variation described

above. On different occasions we have followed the changes shown by *Cl. septicum* in mice for intervals of ten hours, fourteen hours and sixteen hours, as well as for twenty-four hours as reported above. Very similar changes in the morphology of *Cl. septicum* were noted on all of these instances. Furthermore changes very similar to those seen in mice could be demonstrated in pure cultures of *Cl. septicum* when these cultures were made under the proper conditions.

It is of interest to compare our observations on spore-formation with those recently reported by Bayne-Jones and Petrilli (1933). These authors studied carefully the formation of spores in fresh preparations of *Bacillius megatherium* and recorded their findings seven times per minute by means of motion photomicrography. They observed the preliminary development of an area of clearing which seemed to be denser than the rest of the cell protoplasm. The density of this area increased gradually for three or four hours, after which the material in this region contracted rapidly and developed into a characteristic spore in less than thirty minutes. Bayne-Jones and Petrilli concluded that spores did not develop from preformed granules in the organisms that they had studied.

In contrast to the work of the above authors we studied an entirely different organism, our observations were necessarily made at much longer intervals and we examined stained material. The formation of granules preceded the appearance of spores by as much as four hours, although we had no way of observing this in any one organism. Spores definitely appeared to develop from these precedent granules. We can not say, however, that this is the only mechanism of endospore-formation in *Cl. septicum*.

When ordinary cultures of *Cl. septicum* are examined one can tell relatively little about what occurs in connection with the development of spores. But when this organism is put into an environment more nearly approximating its optimum growth conditions, spore-formation and other details of morphology may be observed to greater advantage. It would seem logical to study other organisms under circumstances approximating the conditions for their optimal growth.

SUMMARY AND CONCLUSIONS

Growth changes in *Cl. septicum* were observed following intravenous injection of an eighteen-hour culture of this organism into mice. The animals were killed shortly thereafter and incubated at 37°C. Autopsies were performed at two-hour intervals for twenty-four hours, smears were made from the livers and heart interiors, and photomicrographs were taken. An extreme degree of change in form and staining reaction was noted including the formation of long filaments, the breaking up of these filaments into short segments, the formation of granules within these segments, and the apparent development of spores from these granules.

Similar changes were observed in pure cultures of *Cl. septicum* when grown under suitable conditions.

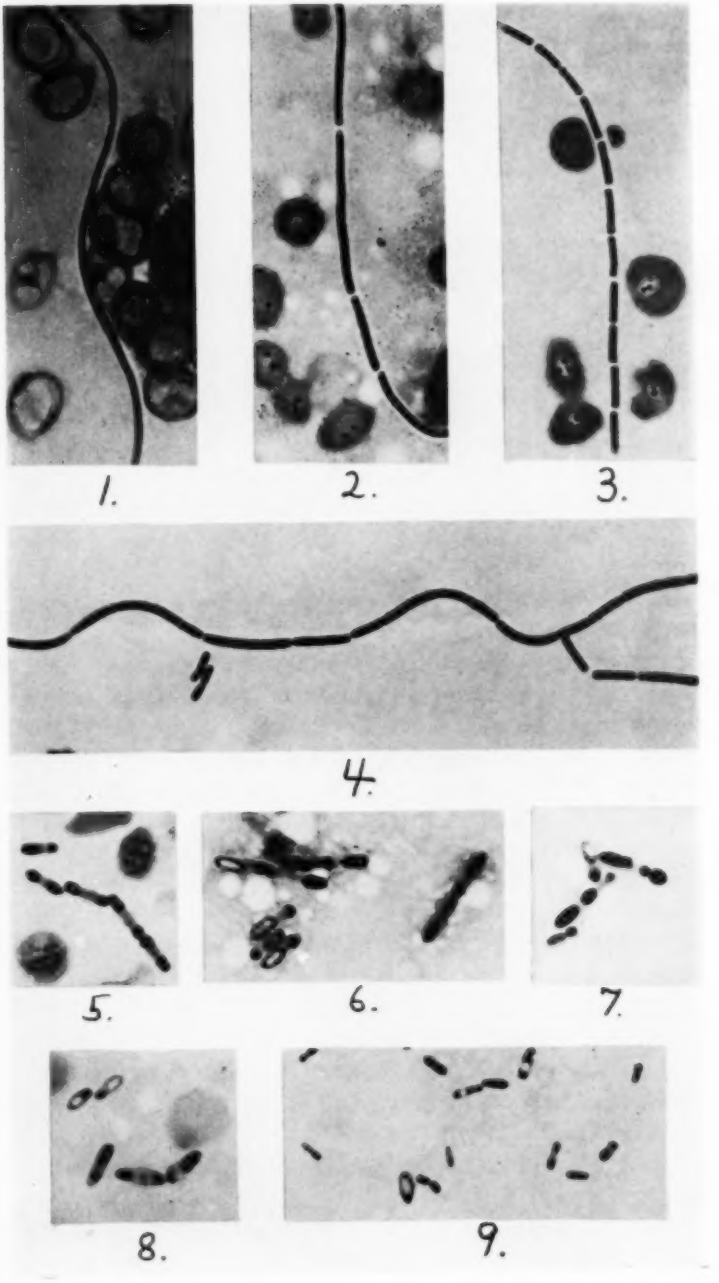
REFERENCE

BAYNE-JONES, S., AND PETRILLI, A. 1933 Jour. Bact., **25**, 261–276.

PLATE 1

All photographs taken at magnification 1200 ×

FIGS. 1 AND 2. *Cl. septicum* from mouse, six hours.
FIG. 3. *Cl. septicum* from mouse, eight hours.
FIG. 4. *Cl. septicum* from serum-chopped-veal infusion broth culture, six hours.
FIG. 5. *Cl. septicum* from mouse, ten hours.
FIGS. 6 AND 7. *Cl. septicum* from mouse, fourteen hours.
FIG. 8. *Cl. septicum* from mouse, eighteen hours.
FIG. 9. *Cl. septicum* from plain chopped-veal infusion broth culture, eighteen hours.



(Anson Hoyt: Growth Phases of Clostridium Septum)

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A BACTERICIDAL PRINCIPLE IN EXCRETIONS OF SURGICAL MAGGOTS WHICH DESTROYS IMPORTANT ETIOLOGICAL AGENTS OF PYOGENIC INFECTIONS[1]

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The elimination of bacteria from pyogenic infections must precede permanent healing. That such a phenomenon occurs under the influence of maggot therapy is well known. Baer, in his original report (1929), called the maggots "a viable antiseptic." Later (1931) he had the following to say: "Whatever the bacteria of the original wound, be they aerobes or anaerobes . . . the amount begins to diminish from the first application of maggots." Weil and coworkers (1933) made preliminary bacteriological studies of wounds before implantation of maggots, followed by subsequent cultures at each dressing, and found a marked decrease in infection as the treatment progressed. Other workers (Buchman and Blair, 1932; Livingston and Prince, 1932; Myers and Czaja, 1931; Slocum et al., 1933) have obtained the same results. How this remarkable phenomenon is accomplished however, has been only partially shown.

Among the factors that contribute to wound disinfection are: 1. The mechanical washing out of bacteria through stimulation of drainage by the maggots; by *a*, mechanical stimulation of the viable tissue; *b*, enzymic liquefaction of the necrotic tissue; and *c*, increase of discharge through dilution by maggot excretions, or a combination of these factors. 2. The destruction of organisms in the alimentary tract of the maggots, as has been shown by Robinson and Norwood (1933). 3. The utilization of necrotic tissue as maggot food, thus rendering conditions less favor-

[1] Division of Insects Affecting Man and Animals, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

able for bacterial growth. 4. The rapid development of vascular granulation tissue. It has also been suggested that the increase in pH is a factor.

The foregoing factors, although of importance, can hardly account for the almost complete sterility obtained in some infections. The excretions of surgical maggots, which are continually released into the wound, have not heretofore been shown to have bactericidal properties. Livingston and Prince (1932) claimed to have demonstrated bactericidal action with filtered extracts from bodies of crushed maggots. Their work, however, was not substantiated by sufficient evidence, and several investigators (Maseritz, 1934; Robinson and Norwood, 1933; Slocum et al., 1933) have since proved their theory to be wrong.

The material used in this investigation is not an extract and is entirely different from that tried by other workers. It consists of the natural elimination products of living maggots, and tests with such material have not been previously reported.

The investigation has revealed that a potent bactericide is present in maggot excretions. The substance collected and used in these experiments consisted of the entire elimination products of the maggots, which included fecal matter as well as cutaneous and oral secretions. There is reason to believe, however, that the active principle is contained in the feces. Robinson and Norwood (1933) found the contents of the hind intestine of maggots to be sterile while those of the crop and stomach were heavily contaminated, and their findings have recently been confirmed by Stewart (1934). Duncan (1926) found that the feces of certain insects were sometimes sterile. The substance at present, therefore, is called "maggot excretions," for lack of a more specific name.

The preliminary work, herein presented, has been confined to tests of the bactericide on seven species of bacteria, *in vitro*. Two of these play the principal etiological rôle in pyogenic infections, and another is often a dangerous and sometimes fatal secondary invader.

All organisms were exposed to the material at a temperature of 37°C., unless otherwise designated. The maggots used were

those of *Lucillia sericata*, the species most commonly employed in wound treatment.

TECHNIC OF COLLECTING EXCRETIONS

Most of the material used was collected from three-day-old non-sterile maggots that had been reared on decayed beef, in order to simulate as closely as possible conditions encountered in maggot therapy. An excess of eggs was placed on the meat, thus increasing the ratio of maggots to food, which facilitated their removal when ready for use. The specimens were mechanically removed by raking them from the residue with a spatula, without the use of water. They were placed in a fine-mesh sieve over a funnel fixed into the neck of a flask. A fine-mesh wire gauze fastened over the top of the sieve by means of a tight-fitting metal collar prevented the maggots from escaping. Distilled water was sprayed occasionally through the top with an atomizer, in order to keep the maggots moist and actively excreting, and also to wash the excretions from the maggots through the sieve into the collecting flask.

The amount of water used varied, and thus, also, the dilution of the excreta. Usually the amount collected was approximately that of the water applied. Collection usually extended over a period of from two to four hours and from 25 to 50 cc. of water were ordinarily used. The quantity of maggots employed varied, but averaged approximately 150 to 200 cc. (measured by jarring the specimens down in a 250-cc. graduated cylinder). After collection, the material was autoclaved at 10 pounds' pressure for twenty minutes, to insure its sterility. It was then placed in the refrigerator at 6°C. and generally used the following day. Each sample was cultured for sterility before use, and negative results were obtained.

As the effect of thermo-sterilization was not known, the first experiments were conducted with excretions collected from maggots that had been reared aseptically. The specimens used were reared as for surgical use (Simmons, 1934 and 1935), washed from their food, and excretions collected as previously described, except for modifications to maintain sterility. This material

was cultured for sterility and used on the day it was collected. It was soon found, however, that the substance could be collected under septic conditions and then sterilized without apparent loss of potency; so the aseptic rearing was discontinued. Excretion from sterile maggots was also considered less typical of that produced by maggots in wounds.

ORGANISMS USED

A large portion of the work was done with a strain of *Staphylococcus aureus*, which is used by the U. S. Food and Drug Administration, for the testing of disinfectants and antiseptics, and has been designated by them as a standard culture which complies exactly with the specifications outlined for the normal resistance of this organism (Ruehle and Brewer, 1931).

Several strains of *Streptococcus*, both haemolytic and nonhaemolytic originally isolated from various sources such as empyema, throat infections, feces, and a knee infection were used.[2] As *Staphylococcus aureus* and *Streptococcus pyogenes* are the principal etiological agents of pyogenic infections, they received major consideration in this investigation.

Tests against *Clostridium Welchii* were conducted with a strain isolated from a fatal case of gangrene following a compound fracture of the leg. Guinea pigs inoculated with this strain were killed, with typical lesions, while antitoxin-protected controls remained alive.

Inasmuch as suppurative infections usually show a high count of *Proteus*, which are the last organisms to disappear from healing wounds, a brief test was conducted to determine the value of maggot excretions against two strains of *Proteus vulgaris*.

In addition to these typical bacteria of pyogenic infections, a test was conducted with a standard strain of *Eberthella typhi* (Ruehle and Brewer, 1931), as this is an organism much used in testing disinfectants and is sometimes important in suppurative infections.

[2] Most of the organisms used, and the information concerning them, were obtained through the courtesy of Major H. R. Livesay of the Bacteriological Department of the Army Medical School, Washington, D. C.

The bactericidal properties of the material against other and more varied forms of organisms, and its clinical applications, are to be investigated. The tests reported in this paper were conducted primarily to show the effect of maggot excretions in the disinfection of wounds.

TESTS ON STAPHYLOCOCCUS AUREUS

With excretions from contaminated maggots

The first tests with *Staphylococcus aureus* were made with cultures of various ages, an unknown number of which were removed from agar slants and suspended in physiological saline. Of the suspension, 1 cc. was placed in 9 cc. of the extract and from

TABLE 1

Effect of heat-sterilized maggot excretions on S. aureus of various ages and densities

	MINUTES' EXPOSURE						
	5	10	30	60	120	180	240
Saline control	+	+	+	+	+	+	+
Excretions	−	−*	−	−	−	−	−

+, bacterial growth; −, sterility.

* One plate showed 20 colonies after seven days' incubation.

this 1:100 and 1:1,000 dilutions were made. Identical suspensions were made in normal saline to serve as controls. These suspensions were incubated at 37°C., and plates made at various intervals, 1 cc. being used for each plate. A summary of eight complete tests is shown in table 1.

Organisms smeared on an agar plate containing 1 cc. of the material showed good growth, proving that death occurred during exposure in the tubes and that the quantity of excretions transferred to the plates would not have inhibited the growth of viable bacteria.

The results obtained indicate that maggot excretions have a strong and rapid disinfectant action on *S. aureus*.

After definitely proving the bactericidal properties of the excretions, tests were conducted on broth cultures of the standard

strain of *S. aureus*, which had been transferred to new broth every twenty-four hours for several weeks. Such cultures appeared to be more resistant to the action of the material.

A 0.1 cc. suspension of a twenty-four hour broth culture of *S. aureus*, containing approximately 50,000,000 organisms, was added to 5 cc. of the excretions and also to 5 cc. of saline. These were incubated at 37°C., and both broth and plate cultures were made at various intervals. The plate cultures of the excretions were negative in every instance, while the results obtained from two tests with the broth cultures are shown in table 2.

This test was repeated with approximately 250,000,000 organisms to 5 cc. of material. Growth was obtained at fifteen minutes, but at 60 minutes all cultures of the excretions were

TABLE 2

Effect of excretions on twenty-four-hour broth cultures of the standard strain of S. aureus

	MINUTES' EXPOSURE				
	5	15	60	120	300
Saline control	+	+	+	+	+
Excretions	+	±	−	−	−

+, heavy growth; ±, light growth; −, negative.

negative, as was the case when only 50,000,000 organisms were used. Since no cultures were made at intervals between fifteen and sixty minutes, it is possible that the less dense suspension was killed in a shorter time than the heavy one. The number of viable organisms after five- and fifteen-minute exposures was evidently small, as was indicated by absence of growth on agar plates.

One test was conducted by the agar cup-plate method. A depression was made in an agar plate thickly sown with *S. aureus*, and a small amount of the excretions placed in the cup. A clear zone of about 1 mm. surrounded the cup after twenty-four hours' incubation. Beyond this zone the substance was apparently too dilute for fatal action.

One test was conducted at 20°C. to determine if bactericidal

value was affected by temperature. Of a twenty-four hour broth culture containing approximately 50,000,000 organisms of the standard *S. aureus*, 1 cc. was subjected to 5 cc. of the excretions. Broth cultures made after fifteen minutes' exposure showed heavy growth. Another culture was not made until after two hours' exposure, at which time a very feeble growth was indicated. One test is hardly sufficient to permit definite conclusions to be drawn, but the indication is that the bactericidal potency diminishes with the temperature, a well-known phenomenon with many disinfectants.

With desiccated excreta

Of excretions from non-sterile maggots, 30 cc. were desiccated under reduced pressure at 60°C. for four hours on the day of collection and for six hours on the following day. The residue, which was still moist, was then placed in an open Petri dish and dried in an oven at 45°C. for twenty-four hours. The material was placed in the refrigerator at 6°C., allowed to remain for four days, and then diluted with 30 cc. of distilled water (the amount of liquid originally present). It was then autoclaved at 10 pounds' pressure for twenty minutes. Much of the material failed to go back into solution.

To 4.5 cc. of the supernatant fluid, placed in a test tube, 0.5 cc. of a light suspension of *S. aureus* was added. Plates made with 0.5 cc. of material at intervals of from ten minutes to two hours showed sterility in every instance, while the saline checks always showed growth. No further attempts at desiccation were made, but this test shows the possibility of obtainng the active principle in a dry powdered form.

With excretions from sterile maggots

Under the conditions involved in these tests, excretions from sterile specimens seemed to be less potent than those from non-sterile maggots. It is possible that the dilutions of the excreta from sterile maggots were greater and that the active principle maintained a constant potency. Evidence indicates the reverse, however. Several tests with this material always showed defi-

nite bactericidal effects on approximately two-week-old cultures of the standard *S. aureus*. In some instances a complete kill was obtained in ten minutes. In most cases, however, plated portions of the excretions showed some growth, but always definitely less than did the controls. Some food in which the maggots had fed was dissolved in sterile water and tested. This also showed definite bactericidal value, but not a complete kill, even at four hours.

The bactericidal action of the maggot excretions on *Staphylococcus aureus* explains in part the rapid diminution in numbers of this organism in infections under maggot therapy, as this material is constantly being discharged into the wound by the maggots.

TESTS ON STREPTOCOCCI

Haemolytic streptococci

The organisms used were haemolytic forms of *Streptococcus pyogenes*. A saline suspension of a strain isolated from a case of empyema was made from blood-agar slants and a 1:10 dilution made from this. Of each suspension 1 cc. was subjected to 9 cc. of the excretions for periods ranging from five minutes to three hours, and plates made at such intervals were always sterile. Saline checks, however, always showed growth.

Another test was conducted with the same strain of organism which had been grown in glucose broth neutralized with $NaHCO_3$. In this test a three-day-old solution of the material was used. A sample of 0.1 cc. of a twenty-four-hour broth culture, containing about 50,000,000 organisms, was exposed to 5 cc. of the excretions, and broth cultures made after five and fifteen minutes' exposure showed no growth.

A twenty-four-hour glucose-broth culture of *S. pyogenes-haemolyticus*, isolated from a throat infection, was next tried. A 0.5 cc. sample of a dense culture was subjected to 5 cc. of fresh excretions, and from this broth cultures were made at intervals of five minutes to two hours. Complete sterility was obtained in every instance. This test was repeated with a sample of material that had been kept in the refrigerator at 6°C. for three

days, and identical results were obtained. The excretions had lost some of their potency against *S. aureus* on standing, however, indicating that the streptococci were even less tolerant to its action than *Staphylococcus*.

Non-haemolytic Streptococci

Tests were conducted to determine if the viridans type of streptococcus was equally susceptible to the action of the excretions. Glucose-broth suspensions of a twenty-four-hour culture of *Streptococcus faecalis* were made from blood-agar slants, and 0.1 cc., containing approximately 50,000,000 organisms, was exposed to 5 cc. of fresh material. Cultures were made at intervals of five minutes to two hours. The five-minute cultures showed growth, but the fifteen-minute exposures were negative, as were all the rest.

This test was repeated with *Streptococcus mitior*, isolated from fluid aspirated from an infected knee, and the same results were obtained. A test with a mixed culture was conducted, in which 0.1 cc. of a twenty-four-hour glucose-broth culture of *S. faecalis*, containing about 50,000,000 organisms was used with a similar culture of *S. mitior* in 5 cc. of a three-day-old sample of excretions. The results were the same as those obtained with pure cultures. Death occurred in fifteen minutes, even though twice as many organisms and twice as much organic matter were added.

In view of these tests, the disappearance of *Streptococcus* from infected wounds under maggot therapy, as with staphylococci, can be attributed in a large measure to the action of the maggot excretions. Such action by this material in turn accounts for much of the gratifying results obtained with such therapy.

TESTS ON CLOSTRIDIUM WELCHII

Clostridium Welchii is a serious and sometimes fatal secondary invader of osteomyelitis and other infected wounds. Therefore, tests with this organism were considered of importance. The principal work was conducted with the strain previously described.

One cubic centimeter of a twenty-four-hour broth culture of

Cl. Welchii was exposed to 5 cc. of material, and 0.1 cc. of this inoculated into Robertson's chopped-meat media at intervals of from five minutes to four hours. No indication of growth from the treated organisms was apparent after twenty-four hours' incubation of the broth, while the saline checks showed heavy contamination. Subcultures were then made into litmus milk and reincubated, but no growth appeared, showing complete kill of the organism in five minutes.

A similar test was conducted with spores of a different strain of *Cl. Welchii* which had been dried in dirt. Some growth occurred even after a twenty-four-hour exposure, but death was indicated at forty-eight hours. From the viewpoint of wound disinfection, however, the resistance of *Cl. Welchii* spores is of little significance, as they are not usually encountered in such lesions.

The remarkable results obtained in the destruction of this organism with maggot excreta offers a possible explanation for the rapid clearing up of gas gangrene infections under maggot therapy, which has been observed by Baer (1931), Weil (1933), and others.

TESTS ON PROTEUS VULGARIS

Since *Proteus* is usually the last organism to disappear from infected wounds under maggot treatment, it was suspected that it might possess a higher resistance to maggot excretions than the other organisms involved. This, however, was found not to be true.

A small quantity of broth, containing at least 50,000,000 *Proteus vulgaris*, was subjected to 5 cc. of material. Plate and broth cultures made at intervals of five minutes to four hours always showed sterility. The test was repeated with identical results.

The persistence of *Proteus* can probably be attributed to its superficial position in the wound, the organism not being abundant in the deeper cavities, where the excretions of maggots naturally collect. The tests show, however, that when subjected to the action of the excretions, this organism shows slight resistance, being killed in as little as five minutes.

TESTS ON TYPHOID BACILLUS

Eberthella typhi being a standard test organism and sometimes of importance in osteomyelitis, a test was conducted[3] to determine its resistance to the excretions. The strain used was one standardized by the Food and Drug Administration of the United States Department of Agriculture for the testing of disinfectants (Ruehle and Brewer, 1931). A quantity of 0.1 cc. of a dense twenty-four-hour broth culture was subjected to 5 cc. of the material for intervals ranging from five minutes to one hour. Cultures at five minutes showed some growth, but complete sterility was obtained in fifteen minutes. Only one test was conducted, but the clear-cut results are indicative of the activity of maggot excretions against this organism.

NATURE OF THE ACTIVE PRINCIPLE

Little is known concerning the nature of the active bactericidal principle of maggot excretions. It is, however, no doubt purely nonviable. Its resistance to heat (110°C. for twenty minutes) indicates the absence of a bacteriophage or an actively bacteriolytic enzyme. It is a nonlytic agent, and apparently bears no relation to bacteriolytic elements found in tears, sputum, nasal mucus, blood serum, etc. The absence of lysis was indicated by the fact that a sample of material contaminated with a haemolytic strain of *Streptococcus pyogenes*, and from which no growth could be obtained, showed, after two days' exposure, when stained the presence of numerous dead bacteria. Duncan (1926) found that the gut content dissected from certain adult insects was devoid of any phage or lytic properties, although he was able to demonstrate bactericidal action.

The effect of dilution, beyond that which took place in collection of the material, is not yet definitely known. A sample of excreta was diluted with equal parts of 0.85 per cent saline, with no appreciable effect on its potency against *S. aureus*, whereas dilution with sterile distilled water caused a considerable drop in bactericidal action.

[3] Through the courtesy of C. M. Brewer, bacteriologist of the U. S. Food and Drug Administration, who was also kind enough to confirm certain other tests presented in this paper.

The apparent difference in activity caused by dilution with saline and with plain water might be due to some physical change.

The indications are that the excretions could be made more potent by using saline during their collection.

The durability of the principle is not known. Its potency diminishes gradually on standing in aqueous solution, but it was found to kill haemolytic streptococci in five minutes after standing for three days. Good bactericidal activity was retained for a week in dried excreta. It is interesting to note that Duncan (1926) found dried dissected gut-contents of certain insects to be as potent as fresh material after six months.

The excretions give an alkaline reaction to litmus, turning neutral paper slightly blue.

Although no tests were made, it is indicated by Duncan (1926) working along somewhat similar lines, that the active principle is tied up with minute solid particles suspended in the solution. He centrifuged and filtered through filter paper a suspension of the gut-content of a tick, *Argus persicus* Fischer, which he had previously proved to have bactericidal properties, and found that the filtrate had lost much or all of its activity. Maseritz (1934), using intestinal emulsions of surgical maggots, failed to obtain any bactericidal results. This could have been due to the fact that he used only filtered samples of emulsions of septic maggots. His failure with such material strengthens the theory put forth by Duncan.

The action of the material simulates that of phenol and similar organic disinfectants. This similarity is somewhat indicated by its poor inhibitory properties, and by the fact that it killed the organisms used in their order of resistance to such agents.

Duncan also found that the bactericidal element in the gut-content of certain insects is not of a lipoid nature, being immune to extraction by acetone, chloroform, and alcohol. He found that dried specimens of gut-content contained a large amount of insoluble material, as has been shown in the writer's experiments, and that this could be liberated by tryptic digestion and thus increase the activity of the specimen.

DISCUSSION

The foregoing experiments demonstrate that wound disinfection by surgical maggots may be due primarily to their excretions, which contain a potent bactericide. It is well known, of course, that *in vitro* tests are not always borne out *in vivo*, but the undeniable fact that remarkable destruction of bacteria occurs under the influence of maggots shows that the practical application of this substance gives positive results.

The remarkable activity displayed against both *Staphylococcus aureus* and haemolytic streptococci no doubt accounts in large part for the success of the maggot treatment of pyogenic infections, as these are the two most important etiological agents. Maggots, however, accomplish more than disinfection of the wound. Robinson (1935) has recently shown that their excretions contain allantoin, the chief end product of purine metabolism, which he has proved to be an excellent cell proliferant. Any cell proliferant would seem to benefit, however, if a bactericide were present to destroy pyogenic bacteria and make way for unobstructed action. The combination of two such factors makes for harmony, and both are present in maggot excretions. The natural coexistence of a substance that causes proliferation of soma cells with a substance that is destructive to bacterial cells is a unique phenomenon.

The good results obtained by both Baer (1931) and Weil (1933) in the treatment of gas gangrene infections by maggots is probably explained by the fatal action of the excretions on *Clostridium Welchii*. In view of these tests, and of *in vivo* tests conducted by others, it appears logical to regard the development of *Cl. tetani* in wounds as the principal remaining infection to be feared. The excretions have not yet been tried against this organism, but it is probable that at least the vegetative form would be killed. Baer (1931) however, had cases of tetanus develop in patients under maggot treatment, which indicates that such infections do not respond to the treatment so readily as gas gangrene. It is possible that the organisms were immune to the excretions, or that they were in inaccessible

cavities. The death of other organisms, elimination of necrotic tissue, and the formation of clean, healthy granulation tissue, however, tend to diminish anaerobic conditions and make less likely an infection of organisms with which maggots could not cope.

The perfection of a non-toxic, non-irritating disinfectant for internal use has not yet been realized. The apparent nature of the material here studied suggests the possibility that some form of it could be utilized in infections where maggots can not be employed.

The material is not recommended for use in the place of maggots, as they accomplish many more things than the disinfection of the wound, as has been pointed out. The investigation was made to show the rôle that maggot excretions play in wound disinfection rather than as an attempt to replace them.

From the tests described herein, it is the author's belief that intensive investigation of bactericidal substances produced by living organisms is a fertile field in which new substances might perhaps be isolated to cope with situations which do not yield to ordinary disinfectants.

SUMMARY

1. A potent bactericide has been obtained from surgical maggots of the species *Lucilia sericata*, and the technic for its collection is described.

2. Bactericidal tests with this substance were conducted with seven species of bacteria of etiological importance in pyogenic infections. The results showed that five- to ten-minute exposures were usually sufficient to give 100 per cent kill of dense saline and broth suspensions of the organisms.

3. The addition of organic material apparently has less effect on the potency of this material than on that of ordinary disinfectants.

4. The active principle is of a nonviable nature, and is not destroyed by autoclaving for twenty minutes at 10 pounds' pressure.

5. No indication of lysis could be demonstrated, and the

thermostability and other reactions of this substance rule out the possibility of a bacteriophage as the active principle.

6. The material was desiccated, and in this dry condition it apparently maintains its potency over a longer period than when in aqueous solution.

7. The remarkable bactericidal potency of the excretions against *Staphylococcus aureus*, haemolytic streptococci, and *Clostridium Welchii* accounts in part for the gratifying results obtained in such infections under maggot therapy.

8. The investigation reveals a field with potentialities of producing other new and useful disinfectants from living organisms.

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THE ANTIGENS OF SALMONELLA ANATUM[1]

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Rettger and Scoville (1920) isolated a number of cultures of paratyphoid bacilli from a fatal disease of young ducklings. These organisms were described and characterized as *Bacterium anatum*, N.S. Further study of these cultures by Edwards and Rettger (1927) revealed that they constituted two distinct types, one identical with *Salmonella aertrycke*, the other an apparently independent type of paratyphoid bacillus unlike any of the cultures with which it was compared. The name, *Salmonella anatum*, was retained for the latter type. Edwards (1929) isolated *S. anatum* from an outbreak of paratyphoid infection in young chicks. Again the organism was found to be associated with *S. aertrycke*. Gaiger and Davies (1930) isolated an organism which they referred to as *S. anatum*, from young chicks. According to Lovell (1932) the organism isolated by Gaiger and Davies was not *S. anatum*, but *Salmonella enteritidis*.

While *S. anatum* had gained recognition as a distinct type of *Salmonella*, no detailed report of its antigenic relationships was published until Lovell (1932) tentatively assigned it an antigenic formula based on the scheme of White (1926). Lovell stated that *S. anatum* possessed the stable antigen of the *S. enteritidis* group or of the London type of Salmonella. This conflict of evidence probably arose through certain English workers mistaking *S. enteritidis* for *S. anatum*. Lovell represented the specific phase of *S. anatum* as similar to the specific phases of the

[1] The investigation reported in this paper was conducted in connection with a project of the Kentucky Agricultural Experiment Station, and is published by permission of the Director.

Newport and Reading types, with the possibility that *S. anatum* possessed additional specific factors. The non-specific phase of the organism was given as that of *Salmonella suipestifer*, with the possibility of the presence of additional factors.

Since some doubt existed as to the exact relations of *S. anatum*, it was thought worth while to determine more exactly the antigenic structure of the species. During the course of the work a paper by Kaufmann and Silberstein (1934) appeared in which were given the results of their work with *S. anatum*. These investigators included in this species three cultures isolated from cases of gastro-enteritis and food poisoning in man. This is the first record of the isolation of this type of *Salmonella* from man. Further reference to the work of Kaufmann and Silberstein will be made later in the paper.

MATERIALS AND METHODS

The cultures used in the investigation were as follows:

S. anatum C1, C2, C5—isolated from ducklings in 1918 by Dr. L. F. Rettger. See Rettger and Scoville (1920).

S. anatum MC2—isolated from baby chicks, 1928.

S. anatum 7089—7089, *S. anatum* var. *muenster*, Natl. Coll. type Cultures, London.

Salmonella, London type 1946, 1946, *Salmonella L*, LII. Natl. Coll. Type Cultures, London.

Salmonella Newport type 1857, 1857, *S. newport*, Slade. Natl. Coll. Type Cultures, London.

Salmonella, Kunzendorf type 1713–1713, *S. Kunzendorf*, Alfonso. Natl. Coll. Type Cultures, London.

Salmonella, Senftenberg type 4165–4165, *S. senftenberg*. Natl. Coll. Type Cultures, London.

Salmonella, Reading type 316–316 Reading. From Dr. Georgia M. Cooper.

S. abortus-ovis H—*B. abortus-ovis* from Dr. H. Miessner, Hannover.

In the characterization of the organisms and in the definition of antigenic factors the recommendations of the Salmonella Subcommittee of the Nomenclature Committee of the International Society for Microbiology (1934) have been followed.

The methods used in the preparation of antigens for agglutination and absorption tests were largely those recommended by White (1926) and Lovell (1932). In testing for the presence of flocculating agglutinins, formalinized broth cultures were used, since it was found that they were much more sensitive to the action of flocculating agglutinins than most suspensions of agar-grown organisms. Considerable difficulty was encountered in preparing agar-grown suspensions which would flocculate quickly in the presence of appropriate antiserums. This was true even when motile colonies were picked from semi-solid agar and sown on moist, freshly prepared agar. Broth cultures flocculated in a few minutes at 50°C. in the presence of antiserum. The reaction was practically completed in two hours, whereas many agar-grown suspensions had only just started to flocculate in that length of time. A further advantage in the use of formalinized broth cultures is the fact that they do not react to somatic agglutinins to any appreciable extent. There is no difficulty in distinguishing floccular from granular agglutination, as there may be when agar-grown antigens are employed.

In the preparation of antigens for the detection of somatic agglutinins the alcohol-treated antigens of White (1926) were used. White's methods of isolating specific and non-specific races, preparation of suspensions for agglutinin absorption, preparation of antiserums, and conducting agglutination tests were followed closely.

RESULTS

Somatic antigens. The results obtained in the study of the somatic antigens are given in table 1. The cultures C5, MC2 and 7089 possess the same somatic factors as the London type. These cultures all possess the factor III of the Senftenberg type as well as the distinctive factor X. Strains C1 and C2 possess the factor III of the Senftenberg type but in them the factor X of the London type is lacking. In its place is a well-developed factor which, as far as could be determined, is not possessed by any of the known salmonellas. This factor is designated as XII.

Specific Antigens. In preliminary tests it was found that, as

Lovell (1932) stated, the specific phase of *S. anatum* closely resembled the specific phases of the Reading and Newport types. Tests to determine definitely the specific factors of strains C1

TABLE 1

Somatic antigens of S. anatum

ANTIGENS	SERA												
	S. anatum C1	*S. anatum* MC2	*London* 1946	*S. anatum* C1 absorbed by *Senftenberg* 4165	*S. anatum* C1 absorbed by *S. anatum* MC2	*S. anatum* MC2 absorbed by *Senftenberg* 4165	*S. anatum* MC2 absorbed by *S. anatum* C1	*S. anatum* MC2 absorbed by *S. anatum* var. *muenster* 7089	*S. anatum* MC2 absorbed by *London* 1946	*London* 1946 absorbed by *Senftenberg* 4165	*London* 1946 absorbed by *S. anatum* C1	*London* 1946 absorbed by *S. anatum* var. *muenster* 7089	*London* 1946 absorbed by *S. anatum* MC2
S. anatum C1....	2000	1000	1000	1000	1000	0	0	0	0	0	0	0	0
S. anatum C2....	2000	1000	2000	2000	1000	0	0	0	0	0	0	0	0
S. anatum C5....	1000	2000	2000	0	0	500	1000	0	0	1000	500	0	0
S. anatum MC2..	2000	4000	4000	0	0	2000	2000	0	0	1000	1000	0	0
S. anatum var. *muenster* 7089..	1000	2000	2000	0	0	500	500	0	0	1000	1000	0	0
London 1946.....	1000	2000	4000	0	0	1000	1000	0	0	1000	1000	0	0
Senftenberg 4165.	1000	1000	1000	0	0	0	0	0	0	0	0	0	0

Figures indicate highest dilution showing definite agglutination.
0 indicates no agglutination at 1:100.

TABLE 2

Specific antigens of S. anatum

ANTIGENS	SERA								
	S. anatum C1 specific	*S. anatum* MC2	*Newport* 1857 specific	*S. anatum* C1 specific absorbed by *Newport* 1857 specific	*S. anatum* C1 specific absorbed by *Reading* 316 specific	*S. anatum* MC2 absorbed by *Newport* 1857 specific	*S. anatum* MC2 absorbed by *Reading* 316 specific	*Newport* 1857 specific absorbed by *S. anatum* C1 specific	*Newport* 1857 specific absorbed by *S. anatum* MC2 specific
S. anatum C1 specific.....	32000	4000	8000	200	100	0	0	0	0
S. anatum MC2 specific.....	32000	4000	8000	100	100	0	100	0	0
S. newport 1857 specific.....	32000	4000	8000	0	0	0	100	0	0
S. reading 316 specific.....	32000	4000	8000	0	100	0	0	0	0

and MC2 are given in table 2. Since the same results were obtained with cultures C2, C5 and 7089 they are not included in the table.

It will be noted that *S. anatum* completely removed the specific agglutinins from the Newport serum. Likewise Newport completely removed the specific agglutinins from *S. anatum* MC2 serum. In absorption of *S. anatum* C1 serum by Newport and Reading and in the absorption of *S. anatum* MC2 serum with Reading, small residues of agglutinins for *S. anatum* remain. These are thought to be due to the presence of small amounts of group components present in the reagents employed. The se-

TABLE 3

Non-specific antigens of S. anatum

ANTIGENS	SERA											
	S. anatum C1 non-specific	*London* 1946 non-specific	*Kunzendorf* 1713	*S. anatum* C1 non-specific absorbed by *Kunzendorf* 1713	*S. anatum* C1 non-specific absorbed by *S. anatum* var. *muenster* 7089 non-specific	*S. anatum* C1 non-specific absorbed by *London* 1946 non-specific	*Kunzendorf* 1713 absorbed by *S. anatum* C1 non-specific	*Kunzendorf* 1713 absorbed by *S. anatum* var. *muenster* 7089 non-specific	*Kunzendorf* 1713 absorbed by *London* 1946 non-specific	*London* 1946 non-specific absorbed by *Kunzendorf* 1713	*London* 1946 non-specific absorbed by *S. anatum* var *muenster* 7089 non-specific	*London* 1946 non-specific absorbed by *S. anatum* C1 non-specific
S. anatum C1 non-specific	8000	20000	8000	2000	1000	0	0	0	0	2000	1000	0
London 1946 non-specific	4000	20000	8000	1000	1000	0	0	0	0	2000	2000	0
Kunzendorf 1713	2000	20000	8000	0	0	0	4000	4000	4000	0	0	0
S. anatum var. *muenster* 7089 non-specific	4000	20000	8000	0	0	0	4000	0	2000	0	0	0
S. abortus-ovis H	4000	20000	8000	1000	1000	0	0	0	0	1000	1000	0
Newport 1857 non-specific	2000	5000	2000	0	0	0	2000	1000	2000	0	0	0

rums were shown to possess a slight amount of group agglutinin after absorption and the antigens were not absolutely specific.

Non-specific Antigens. Preliminary tests revealed that the group factors of *S. anatum* were closely related to those of the London and Kuzendorf types. The results obtained in their study are given in table 3. It may be seen that strain C1 possesses non-specific factors identical with the London type and with *S. abortus-ovis*, as evidenced by mutual absorption. Cultures C2, C5 and MC2 also possess these factors. The formula for the group factors of these cultures is 1, 4, 6.

Culture 7089 is lacking in a factor common to C1, C5, MC2, and London. This factor is also absent in Kunzendorf and therefore must be factor 6. Strain 7089 possesses a factor in common with Kunzendorf which is lacking in C1, C5, MC2, London and Newport. This must be the factor 5. The formula of the nonspecific phase of 7089, then, is 1, 4, 5.

DISCUSSION

The facts pertaining to the antigenic analysis of the Salmonellas have been so thoroughly investigated by the English and Continental workers that there is little need to discuss the present work. However, one point calls for comment. Earlier in the paper it was stated that Kaufmann and Silberstein (1934) published an antigenic analysis of *S. anatum* while the present work was in progress. The results obtained here are in agreement with the conclusions of Kaufmann and Silberstein regarding the labile or flocculating antigens. However, we cannot agree with their conclusions regarding the stable antigens. Kaufmann and Silberstein found that all of their cultures except strain C1 had the stable complex possessed by the London type. The presence of these antigens in cultures C5, 7089 and MC2 has been confirmed. The last-named culture was not studied by the European workers. They found that culture C1 possessed the factor III in common with the London and Senftenberg types, and in addition exhibited a "poorly-developed, apparently rough antigen." In the present work it has been demonstrated that there is a well developed independent antigen in strain C1. Culture C2, which was not studied by Kaufmann and Silberstein, possesses the same independent antigen as strain C1.

In the opinion of the writer the independent factor in cultures C1 and C2 is not due to roughness. The form of growth in broth, colony formation, and saline stability of the *S. anatum* cultures were examined. In testing saline stability the methods of White (1926) and Wilson (1930) were used. Cultures C1 and C2 exhibited less tendency to roughness than the other *S. anatum* strains. Only one culture, MC2, was decidedly rough. This culture produced flat, irregular colonies, formed a pellicle and pro-

duced copious sediment in broth, and was much less stable in high concentrations of salt than the other strains. Yet the rough culture, MC2, apparently possessed the same stable antigens as London, C5 and 7089. In addition, the cultures C1 and C2 do not exhibit the "serological cosmopolitanism" attributed by Schütze (1921) to rough variants. It seems obvious, therefore, that it is erroneous to attribute the independent antigens of strains C1 and C2 to roughness. Since the factor XI has been used by Smith (1934) to denote the stable complex of the Aberdeen type, the factor XII has been applied to the independent antigen of *S. anatum*, C1 and C2. The antigenic formulae of the organisms studied is as follows:

	SOMATIC ANTIGENS	FLOCCULATING ANTIGENS	
		Specific	Non-specific
S. anatum C1, C2	III, XII	eh	1, 4, 6
S. anatum C5, MC2	III, X	eh	1, 4, 6
S. anatum var. *muenster*	III, X	eh	1, 4, 5

SUMMARY

Salmonella anatum possesses the specific antigens of the Reading and Newport types of *Salmonella*. With the exception of one culture studied, the non-specific factors of *S. anatum* were identical with those of the London type and of *S. abortus-ovis*. The non-specific antigens of one culture more closely resembled those of the Kunzendorf type. In three of the cultures the somatic antigens were identical with those of the London type. Two cultures possessed a well-developed somatic antigen unlike those of any other *Salmonella* studied.

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THE GRAM-POSITIVE NON-SPORE-BEARING ANAEROBIC BACILLI OF HUMAN FECES

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In a previous paper (Eggerth and Gagnon, 1933) the predominance of non-spore-bearing anaerobic bacilli in human feces was reported, and a number of the Gram-negative species were described. As observations on the Gram-positive species were at that time incomplete, they were omitted.

Bergey's *Manual of Determinative Bacteriology* (1934) defines the genus *Bacteroides* as consisting of "motile or non-motile rods without endospores. Show good growth with ordinary culture media; without pigment formation. Obligate anaerobes." If this definition is accepted, the Gram-positive species described in this paper fall into the genus *Bacteroides*. For reasons that will appear later, the writer believes that the Gram-negative *Bacteroides* and the Gram-positive non-spore-bearing anaerobic bacilli of the intestine are biologically quite distinct and should be placed in different genera at least; possibly in different tribes or even in different orders. For the present, however, the nomenclature of Bergey's Manual will be followed, as the Manual is the only authoritative system of classification we have. The Gram-positive species will be designated as *Bacteroides*, though it is certain that they will ultimately be placed in another genus.

The organisms to be described were obtained from plate cultures of 85 stools: 11 of these were from breast-fed infants; the remainder, from normal adults. All of them were strictly anaerobic when isolated; with the exception of certain strains of *Bacteroides bifidus*, all have remained obligate anaerobes.

TECHNIC

The technic in general was that of the previous investigation (Eggerth and Gagnon, 1933) to which the reader is referred for more complete details. The feces were first diluted by the method of Torrey (1926); then a 4 mm. loopful of fecal dilution number 4 was well rubbed over the surface of a blood agar plate. This represents an inoculum of 1/3000 mgm. of fresh feces of average water content. Such a plate will usually grow about 100 colonies after five days of anaerobic incubation.

Plating media. For plating the fecal emulsions, a beef-heart infusion agar containing 1.5 per cent of agar, 1 per cent of Parke Davis peptone, and 0.4 per cent of $Na_2HPO_4 \cdot 12\ H_2O$, was used. The pH was set at 7.6, and 5 per cent of sterile blood and 0.15 per cent of sterile glucose were added before pouring the plates. This medium served very well for the original plating of the feces; but it was found that colonies streaked out on a second plate of the same medium sometimes failed to grow. Much better second-generation growth was obtained when liver infusion was substituted for the above heart-infusion agar.

Anaerobic cultivation. Modified McIntosh and Fildes (1916) anaerobic jars were used, as described in the previous paper. Plates were incubated for from five to six days before opening the jars.

Isolation of pure cultures. From three to ten colonies from each stool were selected for pure culture study. Each strain was replated 4 times, using the method of surface streaking; single, well isolated colonies were fished. The liver-infusion blood agar proved very useful for replating.

After a number of strains had been collected and fermentation tests made, some doubt arose as to whether this method actually produced pure cultures. This doubt was occasioned by the fact that the results of the sugar fermentation tests were so very diverse; no two strains agreed completely in their fermentation characteristics, which made classification very difficult indeed. It seemed possible that this great diversity of fermentative characteristics might be due to impure cultures. Therefore many of the strains were subjected to single cell isolation, using a

Chambers micromanipulator and following the technic of Kahn (1929) and of Gee and Hunt (1928). Twenty-eight single cell cultures were obtained, and the fermentation tests were repeated. In each case, exactly the same results were obtained with the single cell cultures as with the original cultures that had been replated 4 times. It would seem, then, that cultures replated 4 times in the manner described actually are pure.

It often proved very difficult to obtain growth from single cells of these anaerobes, and there were many failures. Various media were tried for the initial propagation of the single cells; by far the best for this purpose was a beef-heart infusion broth containing 5 per cent of sterile blood and 2 per cent of glucose.

Stock cultures. These were carried on brain medium, as described before (Eggerth and Gagnon, 1933).

Medium for carbohydrate fermentation tests. This medium had the following composition: 1 per cent of Parke Davis peptone; 0.5 per cent of NaCl; 0.2 per cent of agar; and brom-cresol-purple as acid indicator. The medium was sterilized in small flasks in the autoclave. The carbohydrates (enough to make 1 per cent of the medium) were autoclaved separately in distilled water and added to the flasks; finally, 4 per cent of sterile serum was added. The completed medium was then distributed in 100 by 11 mm. tubes.

It is important to inoculate the fermentation tubes very heavily, otherwise growth may not take place. For this purpose, a nichrome wire with a spiral at the end was used. Young cultures in brain medium served as the source of the inocula. Incubation of the fermentation tubes was continued for forty days, the jars being opened every five to eight days.

For milk medium, a powdered milk (Klim) was used.

Gelatin medium was prepared by adding 10 per cent of gelatin and 0.15 per cent of glucose to phosphate heart infusion broth and adjusting to pH 7.8. After sterilization, 4 per cent of sterile serum was added.

For indol tests, heart-infusion broth was fermented with *Salmonella Schottmuelleri* to remove the sugar.

Lead acetate broth was prepared by adding 1 cc. of sterile 1

per cent lead acetate to 200 cc. of sterile broth containing 0.5 per cent of glucose. Duplicate tubes were prepared containing also 4 per cent of sterile serum.

Coagulated egg-albumen broth was prepared by cutting cubes of coagulated egg white into tubes of infusion broth and sterilizing.

Nitrate broth was prepared by adding 0.5 per cent of sodium nitrate to infusion broth.

One hundred and thirty strains of Gram-positive non-spore-bearing anaerobic bacilli were studied. These were grouped in 11 species, as follows:

1. *Bacteroides lentus* (isolated 23 times; one single cell culture was obtained).

The morphology of different strains of this organism is very variable. In 15 of the 23 strains, the predominating form is a short oval bacillus, 0.5 to 1.5μ broad and 1 to 3μ long; with these, small coccoid forms or long cigar shaped bacilli up to 6μ in length may occur. The bacilli are usually found in chains of 2 to 20 elements; often the bacilli in a chain are set at angles to each other, giving a "rail fence" appearance. In the other 8 strains, the predominating forms are rather slender rods, 0.3 to 0.5μ thick and 1.5 to 3μ long; these may be single, or, more commonly, in long chains. Many of the strains show both the thick oval bacillus and the more slender one, and sometimes both forms occur in the same chain.

A type of cell that is often found is what Tissier (1908) describes as a "peloton de jardinier" (gardener's ball of twine). The middle of the bacillus is swollen, often to 2 or 3 times the diameter of the cell. This cell cannot be described as fusiform or spindle shaped; for the ends are usually not pointed; in fact, they are often slightly thickened. Such a cell resembles a stick with a ball of twine wound around its middle, hence Tissier's simile. These cells usually occur single.

Most of the strains do not show metachromatic granules. In a few strains, these granules are large and numerous, almost filling the entire cell.

This organism is non-motile.

On blood agar and on liver-infusion blood agar the colonies of this species are small, being 0.25 to 0.75 mm. in diameter. They are slightly raised, smooth, and gray. In 3 strains, the colonies show irregular knobs and projections. There is no action on the red cells. As a rule, colonies do not appear on glucose agar, though there is some growth where the inoculation is heavy.

With the possible exception of glucosamine, none of the carbohydrates are fermented by this organism. A slight acidity (pH 6.2 to 6.6) develops in the glucosamine tubes. It seems possible that this is due to the deaminization of the glucosamine rather than to a true fermentation with acid production; this is suggested by the fact that the presence of the glucosamine does not stimulate the growth of this organism, as a fermentable sugar might be expected to do.

Twelve of the 23 strains blacken lead acetate, the others do not.

Only one strain reduces nitrates to nitrites. A single cell culture was obtained of this strain, so it seems quite certain that it is a pure culture.

Indol is not formed, gelatin is not liquefied. There is no action on milk or coagulated egg albumen. Large doses injected subcutaneously into mice and rabbits are without pathogenicity.

2. *Bacteroides ventriosus* (Tissier 1908) (isolated 3 times).

It seems probable that the organisms described by Tissier as *Coccabacillus oviformis* and *Bacillus ventriosus* are identical; the chief differences in Tissier's description are morphological ones, and in dealing with this group of organisms, minor morphological differences cannot be taken too seriously. The writer has chosen the name *ventriosus* rather than *oviformis* because Tissier's description of the former best fits the 3 strains here described.

On blood agar, in glucose serum broth, and in brain medium, this organism appears as a small thin rod about 0.3μ thick and 2μ long. It forms chains of from 3 to 20 members; the chains are often angulated, like a rail fence. The members of the chain may vary greatly in size and appearance; some may be minute cocci; others, normal bacilli; some may be rods with large median swell-

ings, the "peloton de jardinier" of Tissier. On glucose agar the bacilli are more often single, 0.7 to 1.2μ thick and 2 to 4μ long. The organism is non-motile.

On blood agar plates, the colonies are 1 mm. in diameter, smooth, raised, and gray; there is no action on the red cells. Surface cultures on glucose agar show only slight growth where the inoculum is heavy, without visible colonies.

Two of the three strains ferment glucose, levulose, galactose, and mannose. Glucosamine medium is slowly and slightly acidified. The third strain ferments these sugars, and in addition slowly ferments maltose (in thirty-two days). Gas is not formed. No other carbohydrates are fermented.

There is no action on gelatin, milk, coagulated egg white, lead acetate, or sodium nitrate. Indol is not formed. This species is non-pathogenic for white mice and rabbits.

Tissier notes the fact that this organism dies out very readily in cultures. This was also the writer's experience, as all three strains were soon lost through death of stock cultures.

3. *Bacteroides aerofaciens* (isolated 18 times; 5 single cell cultures were obtained).

This organism appears as a bacillus varying in length from 0.8 to 5μ, with a usual length of 2 to 3μ; the thickness varies from 0.4 to 2μ. The ends are rounded or pointed. It forms chains of 2 to 10 elements. The individuals in one chain often vary enormously in size and shape: long bacilli and cocci of various sizes succeed each other in the chain; Tissier's "peloton de jardinier" is common; also geniculate bacilli and bacilli with various irregular projections. Some of the rods are curved, and when several curved forms occur in a chain the chain may be remarkably twisted or knotted.

In 4 of the 18 strains, a few branching forms were found. These were never abundant; they were most often seen in glucose agar cultures, without blood. The branches took the form of short terminal bifurcations. It is probable that the geniculate, nail head, or otherwise irregular forms often seen in this species represent abortive or incomplete branching.

Metachromatic granules are numerous on glucose agar and on potato medium, less common on blood or liver agar.

This organism is non-motile.

On blood agar plates the colonies of this species are 1 to 1.5 mm. in diameter, gray-white, and hemispherical. There is no action on the red cells. On blood liver agar the colonies are 1.5 to 3 mm. in diameter, slightly raised, gray-white, with a more opaque center. On glucose agar the colonies are 1 to 2 mm. in diameter, gray-white; with some strains they are very tough and difficult to emulsify.

In glucose broth a slight turbidity develops; there is also a sediment which may be small in amount and flocculent, or it may be a large loose stringy mass. With most strains the final pH in glucose broth (after fifteen days of incubation) is 4.8.

All of the 18 strains ferment dextrin, galactose, glucosamine, glucose, lactose, levulose, maltose, mannose, sucrose, and trehalose. With aesculin, amygdalin, glycogen, methyl glucoside, salicin, and starch, the results are variable (all but 2 strains ferment starch). None of the strains ferment adonitol, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, mannitol, melezitose, raffinose, rhamnose, sorbitol, or xylose.

Abundant gas is formed from the fermentable sugars. Milk is acidified, but not coagulated; coagulation takes place on heating the milk cultures. There is no action on gelatin, lead acetate, or coagulated egg white. Nitrates are not reduced and indol is not formed.

None of the strains of this species are pathogenic for white mice. Several of them, however, produce subcutaneous abscesses when injected into rabbits; the abscesses heal promptly if incised, more slowly otherwise. The organisms are numerous in the pus, appearing as single slender bacilli, or shorter diplo-bacilli. Tissier's "peloton de jardinier" forms may appear in the pus.

4. *Bacteroides biformis* (isolated 7 times; 1 single cell culture was obtained).

In brain medium and in glucose broth, this organism appears chiefly as a short oval cocco-bacillus 0.7 to 1μ thick and 1 to 1.5μ

long; it occurs single, in pairs, or in chains of varying length. Some strains can easily be taken for streptococci. In glucose broth and on blood agar, fusiform bacilli from 2 to 8μ in length may also be found; these occur in short chains. These long rods are often curved. Some strains show still other variations, especially on blood agar: Tissier's "peloton de jardinier;" also hooked, geniculate, and variously irregular forms. In 2 strains a few definitely branching bacilli were found; the branches were short and were either terminal or lateral. On potato, where growth is very poor, the cells appear as large, very irregular masses that have the appearance of several bacilli fused together. Metachromatic granules may be large and and numerous in the bacillary forms, or they may be absent. The organism is non-motile.

The colonies of this species are characteristic. On liver-infusion blood agar they are flat, grayish, with a wavy outline. They are 3 to 6 mm. in diameter. They may be smooth or they may be studded with small knobs. On blood agar plates the colonies are similar but smaller (1.5 to 3 mm. in diameter). On glucose agar, 5 strains fail to grow in colonies, though there is slight growth where the inoculum is heavy. Two strains form a very few flat colonies on glucose agar, 2 to 3 mm. in diameter.

A slight turbidity develops in glucose broth, with a small flocculent sediment. The pH reaches 5.2 to 4.8.

All 7 strains ferment galactose, glucosamine, glucose, levulose, mannitol, mannose, and trehalose. The results are variable with aesculin, amygdalin, cellobiose, dextrin, glycogen, lactose, maltose, raffinose, sucrose, salicin, and starch. None of the strains ferment adonitol, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, melezitose, rhamnose, sorbitol, or xylose.

Milk is acidified and coagulated by those strains that ferment lactose. There is no action on gelatin, coagulated egg white, lead acetate broth, or nitrate broth. Indol is not formed.

Gas is formed from the fermentable carbohydrates, especially from mannitol; with this alcohol, gas formation is more vigorous than it is from glucose.

This species is non-pathogenic for white mice. When injected

subcutaneously into rabbits, it produces abscesses like those formed by *Bacteroides aerofaciens*. The organisms are numerous in the pus, appearing chiefly as coccoid forms in pairs and chains.

This organism is closely related to the previous species, from which it differs in the following respects:

a. In morphology, it often appears like a streptococcus, especially on brain medium and liver-infusion blood agar.

b. The colonies are much larger and flatter.

c. Mannitol is regularly fermented, which is never the case with *Bacteroides aerofaciens*. Raffinose is fermented by 3 of the 7 strains, whereas it is not fermented by *Bacteroides aerofaciens*.

d. All strains of *Bacteroides aerofaciens* ferment dextrin, lactose, maltose, and sucrose. Only 2 out of 7 strains of *Bacteroides biformis* ferment dextrin, 5 strains ferment lactose, 4 strains ferment maltose, and 4 strains ferment sucrose.

5. *Bacteroides tortuosus* (Debono, 1912) (isolated 3 times; one single cell culture was obtained).

The 3 strains here classified as *Bacteroides tortuosus* differ somewhat from one another. Only one of them fits Debono's description very closely. This is the strain from which a single cell culture was obtained (strain 1).

When strain 1 is grown on brain medium, only single slender bacilli, 0.3 to 0.5μ wide and 2 to 4μ long, appear. On glucose agar, blood agar, and liver-infusion blood agar, they are 0.3 to 0.6μ wide and 2 to 6μ long; here they may occur single, or in pairs or short chains. The chains may occasionally be very tortuous, due to the fact that several successive bacilli in the chain are strongly curved. Smears made from the sugar fermentation tubes when they have become acid often show other forms: extraordinarily tortuous chains; rods with huge median swellings (the "peloton de jardinier"); and bacilli with distinct terminal or short lateral branches. Metachromatic granules may be very numerous in some cells. There is no motility.

On blood agar, the colonies are 3 to 4 mm. in diameter, grayish white, flat, with irregular fimbriated borders. There is no action on the blood cells. On liver infusion blood agar, the colonies are

4 to 5 mm. in diameter, yellowish white, flat, with smooth edges. On glucose agar, the colonies are 2 to 3 mm. in diameter, otherwise as on blood agar.

In glucose broth, a slight turbidity appears, with a flocculent sediment. The pH reaches 4.8.

When strain 2 is grown in brain medium, long chains are formed. The tortuosities which give the species its name are very rare. On liver-infusion blood agar, terminal branchings are frequent. This strain is not very strongly Gram-positive; the Gram stain frequently shows the organism as a Gram-negative bacillus with numerous Gram-positive granules in it.

Strain 3 appears on all media as a slender bacillus, 0.3 to 0.5μ broad, 2 to 3μ long; it is usually single, but may occur in pairs or short chains. Tortuosities and branched forms were never observed.

The colonies of strains 2 and 3 are similar to those of strain 1, except that they are surrounded by a wide zone of hemolysis.

All 3 strains ferment aesculin, amygdalin, cellobiose, galactose, glucosamine, glucose, levulose, maltose, mannitol, mannose, salicin, sucrose, and trehalose. Strains 1 and 2 likewise ferment dextrin, glycogen, lactose, raffinose, and starch; strain 3 does not. None of them ferment adonitol, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, melezitose, rhamnose, sorbitol, or xylose. Abundant gas is formed from the fermented carbohydrates.

Strains 1 and 2 acidify and coagulate milk. Indol is not formed; there is no action on gelatin, nitrate broth, lead acetate broth, or coagulated egg white. This species is non-pathogenic for white mice or rabbits.

6. *Bacteroides catenaformis* (isolated 6 times).

On all the media used, this organism appears as a slender rod, 0.3 to 0.5μ thick, 2 to 3.5μ long, arranged in long chains of 20 to 100 or more elements. Often the bacilli are so closely approximated in the chain that they appear as a continuous filament, no demarcations being evident. Usually the rods are straight, but occasional places will be found where successive bacilli are so strongly curved that the chain is exceedingly twisted as in *Bacte-*

roides tortuosus. The rods in this tortuous portion are often thickened. Another striking feature is the presence of large globular cells, 2 to 3μ in diameter; these may occur in the chains, or, more often, at the ends of the chains, where they may appear pear shaped or club shaped. Occasionally huge sickle shaped cells, 1.5 to 2μ wide at their thickest portion, may occur in the chain. Terminal Y branching may also be found. Metachromatic granules are numerous. The organism is non-motile.

Colonies on blood agar and on liver-infusion blood agar are 2 to 3 mm. in diameter. The edges are flat, but the central portions rise to high, irregular hillocks. The colonies have wavy, irregular edges and are usually radially striated.

In glucose broth cultures a large stringy mass grows at the bottom of the tube, with a clear supernatant fluid. The acidity is low; 10-day cultures in glucose serum broth have a pH of 6.4 to 5.6.

Indol is not formed. There is no action on milk, gelatin, coagulated egg white, nitrate broth, or lead acetate broth. There is no pathogenicity for white mice or rabbits.

Acid, but no gas, is produced by all 6 strains from amygdalin, cellobiose, dextrin, galactose, glucosamine, glucose, glycogen, lactose, levulose, maltose, mannose, raffinose, salicin, and trehalose. The results are variable with aesculin, inulin, mannitol (2 strains out of 6 ferment mannitol), methyl glucoside, methyl mannoside, sucrose (1 strain fails to ferment sucrose), and starch (3 strains). None of them ferment adonitol, arabinose, dulcitol, erythritol, glycerol, inositol, melezitose, rhamnose, sorbitol, or xylose.

7. *Bacteroides pseudoramosus* (Distaso 1912) (isolated 26 times; 4 single cell cultures were obtained).

On blood agar and on liver-infusion blood agar this organism appears chiefly as a slender bacillus 0.2 to 0.4μ thick and 0.8 to 2μ long. It occurs singly, in pairs, or in short chains. The rods have rounded or pointed ends, and are often curved. In glucose broth and on glucose agar, the bacilli are plumper and shorter; they may be oval or wedge shaped; they are often geniculated,

and occasional terminal branching may be observed. Young cultures in brain medium may show bacilli up to 10μ in length. The organism is non-motile.

When first isolated, branching forms of this organism are hard to find, but after prolonged cultivation they may be found more readily, especially on brain medium and glucose agar. One strain (Schafer 1), which is typical in every other respect, now branches freely in brain medium, so that every field in a stained preparation shows definite branching forms.

On blood agar plates the colonies are very characteristic. They are from 0.5 to 2 mm. in diameter, quite markedly elevated, smooth, opaque, either white or yellowish, and are surrounded by a wide zone of hemolysis. On liver infusion blood agar the colonies are from 1 to 2.5 mm. in diameter. Growth is good on glucose agar.

In glucose broth a slight turbidity develops, with a sediment. The pH reaches 4.8.

All strains form acid and a small amount of gas from adonitol, galactose, glucosamine, glucose, glycerol, levulose, mannose, methyl mannoside, sorbitol, and trehalose. Seven out of 26 strains ferment erythritol, 13 ferment mannitol. With dextrin, glycogen, maltose, and starch, the results are very variable: a few strains produce abundant acid; some produce a slight acidity (pH 6.0 to 6.6); most of them fail to ferment. Sucrose and inositol are fermented by one strain only. In the fermentation tubes, lactose is slowly fermented by 5 strains (in thirty to forty days); in milk, however, lactose is fermented by all strains, as the milk reaches pH 5.0 to 5.4. None of the strains ferment aesculin, amygdalin, arabinose, cellobiose, dulcitol, inulin, melezitose, methyl glucoside, raffinose, rhamnose, salicin, or xylose.

Coagulated egg white is not digested. Milk is acidified and coagulated. Gelatin is liquefied. Indol is formed. Nitrates are reduced to nitrites. Lead acetate is feebly and inconstantly blackened.

This organism is not pathogenic for white mice or guinea pigs. When injected subcutaneously into rabbits, abscesses develop. Unless incised and drained, these abscesses persist for weeks;

they eventually heal up completely. The organisms appear in the pus in large numbers as small cocco-bacilli, single or in pairs.

At one time during the progress of this work, the writer believed that this organism was a human pathogen of some importance. It was found repeatedly in various anaerobic cultures, such as those made from abdominal fluid in appendicitis, perinephritic abscess, etc. In a series of anaerobic blood cultures in acute rheumatic fever, it appeared in about 40 per cent of the cultures. However, when the controls were made, it was found that this organism is a very common contaminant. Strange as it may seem, this strict anaerobe is frequently present in the laboratory air. Its presence in cultures must therefore always be looked upon with suspicion. It is even possible that in stool cultures it is a contaminant. The writer believes, however, that *Bacteroides pseudoramosus* has its habitat in human feces, chiefly because no other source could be found. Throat cultures were uniformly negative; so also were cultures of the feces of the horse, dog, cat, rabbit, guinea pig, and white mouse, and of the intestinal contents of the cockroach. Several samples of soil were negative for this organism. Distaso (1912) also found this organism to be very common in human feces.

8. *Bacteroides avidus* (isolated twice; 1 single cell culture was obtained).

This organism appears as a thick bacillus, 0.5 to 1μ broad, 1 to 2.5μ long. The ends are rounded or pointed, and the rod is often slightly curved. Occasional branching forms may be found. Metachromatic granules are few. It is non-motile.

On blood agar and glucose agar plates the colonies are raised, yellowish white, and smooth; they are 2 to 3 mm. in diameter. They are not hemolytic.

Growth in glucose broth is diffuse; the pH reaches 4.8.

Both strains rapidly form acid and a small amount of gas from adonitol, dextrin, erythritol, galactose, glucose, glycerol, inositol, levulose, maltose, mannose, melezitose, starch, sucrose, and trehalose. Both strains slowly acidify glucosamine, inulin, lactose, and raffinose (in twenty to forty days). One strain fer-

ments glycogen, the other does not. Neither strain ferments aesculin, amygdalin, arabinose, cellobiose, dulcitol, mannitol, methyl glucoside, methyl mannoside, rhamnose, salicin, sorbitol, or xylose.

Milk is acidified and coagulated, then partly digested. Cubes of coagulated egg albumen, while not digested away, become completely transparent. Indol is not formed. Nitrates are not reduced. Gelatin is liquefied. Lead acetate is blackened. The organism is non-pathogenic for white mice and rabbits.

9. *Bacteroides limosus* (only one strain isolated; this was obtained as a single cell culture).

This organism is 0.5 to 1.5μ thick and 1 to 5μ long, the usual length being 3 to 4μ. There is much pleomorphism; short ovals, wedge-shaped bacilli, curved, hooked, and bifid forms are numerous. Metachromatic granules are absent. The organism is non-motile.

On blood agar and on glucose agar, the colonies are from 2 to 4 mm. in diameter, raised, and cream colored. They are exceedingly mucoid and adherent.

In glucose broth a diffuse cloudiness with a heavy mucoid sediment appears. The pH reaches 4.8.

Acid and a large amount of gas are formed from adonitol, erythritol, dextrin, glucose, levulose, mannitol, and trehalose. A slight acidity (pH 6.0 to 6.5) without detectable gas slowly develops in aesculin, cellobiose, glucosamine, inulin, glycogen, maltose, mannose, methyl mannoside, raffinose, salicin, starch, and sucrose. No acid is formed in amygdalin, arabinose, dulcitol, galactose, glycerol, inositol, lactose, melezitose, methyl glucoside, rhamnose, sorbitol, or xylose.

Milk is not acidified or coagulated. Lead acetate is not blackened. Gelatin is slowly liquefied. Indol is not formed. There is no action on coagulated egg white or nitrate broth. The organism is non-hemolytic, and non-pathogenic for white mice and rabbits.

10. *Bacteroides cornutus* (Distaso, 1912) (one strain isolated).

Distaso (1912) describes this species as being very common in the feces of human adults. It is strange, then, that only one strain was isolated in this investigation.

Bacteroides cornutus appears chiefly as a slender bacillus, 0.2 to 0.4μ thick and 0.7 to 3μ long. It occurs single or in pairs. In morphology it resembles *Bacteroides bifidus*: bifid, geniculate, and curved forms are common; also the "peloton de jardinier" of Tissier. Metachromatic granules are common. It is non-motile.

A slight turbidity develops in glucose broth, with a granular sediment. The pH reaches 4.8.

On blood agar plates the colonies are from 0.5 to 0.75 mm. in diameter; they are gray, smooth, and hemispherical, and are slightly hemolytic. On liver infusion blood agar, they are from 1.5 to 2 mm. in diameter and are brown in color. Growth was not obtained on glucose agar.

This organism forms acid but not gas from dextrin, galactose, glucose, inositol, levulose, maltose, mannose, trehalose, and xylose. Glucosamine is slightly acidified (pH 6.4). Neither acid nor gas is formed from adonitol, aesculin, amygdalin, arabinose, cellobiose, dulcitol, erythritol, glycerol, glycogen, inulin, lactose, mannitol, melezitose, methyl glucoside, methyl mannoside, raffinose, rhamnose, salicin, sorbitol, starch, or sucrose.

Milk, gelatin, lead acetate broth, nitrate broth, and coagulated egg white show no change. Indol is not formed. The organism is not pathogenic for white mice or rabbits.

11. *Bacteroides bifidus* (Tissier, 1900) (isolated 40 times; 14 single cell cultures were obtained).

In young cultures (18 to 48 hours) on a favorable medium such as glucose serum broth, brain, or chopped liver medium, the organisms appear as rods varying from 0.4 to 1μ in thickness and 0.75 to 8μ in length. The usual length is 2 to 4μ. They occur singly, more rarely in pairs or short chains. Terminal or central thickenings and various irregular projections are very frequent. Most strains show evident branching. The branches are from 0.5 to 1.5μ in length; they may be either terminal or lateral. At

this stage, the organism uniformly retains the Gram stain. Metachromatic granules are very numerous; each bacillus usually contains two terminal granules, and often one or more centrally located. They are usually small, but in some strains large bands or masses occupying a third or more of the bacillary body may stain metachromatically. These granules are conspicuous in the living bacillus as seen in the hanging drop, where they have a distinct reddish coloration.

In cultures four to six days old new features may appear, especially if the culture has become acid. Branched forms may become more abundant (in some strains, however, they become more difficult to find). Cells with as many as 6 to 8 lateral branches may be found in some strains; compound branching may occur. Rods with large bulbous swellings, 2 to 4μ in diameter, may be very numerous. Variously curved, hooked, or tailed forms may appear, also giant bacilli 10 to 12μ long. In some cultures the bacilli become very granular, so that they have the appearance of a chain of close-set, irregular cocci. If the culture has become acid, the Gram stain is taken in an irregular way. Some organisms retain the stain entirely, others will be Gram-negative, and still others will be Gram-negative with Gram-positive granules or bands, variously located. In still older cultures, most or all of the organisms may be Gram-negative. Metachromatic granules are less abundant in the older cultures and may disappear entirely.

The colonies on blood agar plates are from 0.5 to 2 mm. in diameter; they are hemispherical, smooth, or faintly stippled; they are gray in color, glistening, and have a smooth circular outline. The medium is unchanged. On liver infusion blood agar, the colonies are from 0.5 to 3 mm. in diameter; they are hemispherical or conical, smooth or faintly stippled, and glistening; the outline is usually circular but may be slightly crenated. The color of the colonies on liver infusion blood agar varies from white to dark brown; the medium around the colonies is turned brown.

Glucose broth cultures show a slight turbidity with a stringy sediment. The pH after ten days of incubation varies from 4.2 to 5.0.

TABLE 1

Showing the variable fermentation reactions of Bacteroides bifidus

STRAINS	ORIGIN	ARABINOSE	XYLOSE	MELEZITOSE	MANNOSE	METHYL MANNOSIDE	ARBUTLIN	AMYGDALIN	SALICIN	CELLOBIOSE	MANNITOL	SORBITOL	INULIN
Group 1													
Infant 4	Infant's stool	−	−	−	+	+	−	−	−	−	−	−	−
Infant 7	Infant's stool	−	−	−	+	+	+	−	+	+	−	−	+
Michel	Infant's stool	−	−	−	+	+	−	−	−	−	−	−	+
Martin	Infant's stool	−	+	−	+	+	+	−	+	+	−	−	+
Hoff	Infant's stool	−	−	−	+	+	+	−	+	+	−	−	+
Rodwin 3	Infant's stool	−	−	−	+	+	−	+	+	+	+	+	+
Infant 2	Infant's stool	−	−	−	+	+	−	−	−	−	−	−	+
O'Leary 3	Infant's stool	−	−	−	+	+	+	+	+	+	+	+	+
Grotti 1	Infant's stool	−	−	−	+	+	+	+	+	+	+	+	+
Lucille 1	Infant's stool	−	−	−	+	−	−	−	−	+	−	+	−
Larson	Infant's stool	−	−	−	+	+	−	−	−	−	−	−	+
Eisner	Adult stool	−	−	−	−	−	−	−	−	−	−	−	−
Helg. 3	Adult stool	−	−	−	+	−	+	+	+	−	−	−	−
Z 6	Adult stool	−	−	−	−	−	−	−	−	−	−	−	−
J 2	Adult stool	−	−	−	+	−	+	+	+	−	−	−	+
Group 2													
Andy 4	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
E 2	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
Dev. 7	Adult stool	+	+	+	+	−	+	+	+	+	−	+	+
Vogel 1	Adult stool	+	+	+	−	−	−	−	−	−	−	−	+
Vogel 2	Adult stool	+	+	+	−	−	−	+	+	+	−	+	+
Duffy 1	Adult stool	+	+	−	+	+	−	−	−	−	−	−	+
Clem. 9	Adult stool	+	+	+	−	−	−	+	−	−	−	+	+
Winton 1	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
Tiffany 3	Adult stool	+	+	+	+	+	−	−	−	−	−	−	+
Price 1	Adult stool	+	+	+	−	−	−	−	−	−	−	−	+
E A 1	Adult stool	+	+	+	+	+	−	−	−	−	−	−	+
Helen 1	Adult stool	+	+	+	−	−	+	−	+	−	−	+	+
Rogers 1	Adult stool	+	+	+	−	−	−	−	−	−	−	−	+
Dana 4	Adult stool	+	+	+	+	−	+	+	+	+	−	+	+
Dana 7	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
Helen M	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
Z 5	Adult stool	+	+	−	−	−	+	+	+	+	−	−	+
Rotter 3	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
Brown 3	Adult stool	+	+	−	−	−	+	+	+	+	+	−	+
E H	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
O 3	Adult stool	+	+	−	−	−	+	+	+	+	−	−	+
Lucille 3	Adult stool	+	+	−	−	−	+	+	+	+	−	+	+
Gu 3	Adult stool	+	+	−	−	−	−	+	+	+	−	+	+
J 5	Adult stool	+	+	+	−	−	+	+	+	+	−	+	+
E N 1	Adult stool	+	+	−	−	−	+	+	+	+	−	−	+

Milk is acidified by all strains and coagulated by most of them. There is no action on nitrate broth, lead acetate broth, gelatin, or coagulated egg white. Indol is not formed. This organism is non-pathogenic for white mice or rabbits.

None of the 40 strains ferment adonitol, dulcitol, erythritol, glycerol, or rhamnose. Only 2 strains ferment inositol. All strains form acid but not gas from dextrin, galactose, glucosamine, glucose, glycogen, lactose, levulose, maltose, methyl glucoside, raffinose, starch, sucrose, and trehalose.

With the remaining carbohydrates, the results of fermentation tests are variable. On the basis of acid production from arabinose, xylose, melezitose, and mannose, it is possible to divide the 40 strains into 2 groups: group 1 being negative on arabinose, xylose (except for one strain), and melezitose, but positive on mannose; group 2 being positive on arabinose and xylose and usually melezitose, but usually negative on mannose. This grouping acquires considerable significance from the fact that all of the strains from infants' stools fall into group 1, while most of the strains from adults' stools fall into group 2. This classification is brought out in Table 1. To the 15 strains in group 1, one may properly add the 31 strains studied by Weiss and Rettger (1934); these were all negative on arabinose, xylose, and melezitose (their action on mannose was not stated) and they were all isolated from nurslings' stools.

Strain "Lucille 1," given in Table 1, was isolated from the stool of a breast-fed infant; strain "Lucille 3" was isolated from the stool of the same child, but two years later. It is interesting to note that with the change in age and diet, there was a change in the type of *Bacteroides bifidus* found. In the cases of two stools from adults, both types of *Bacteroides bifidus* were isolated from the same stool (Z 5 and Z 6, and J 2 and J 5).

No consistent morphological differences between the two groups of *Bacteroides bifidus* could be discovered. Attempts were made to ascertain the serological relationship of these two groups, but with unsatisfactory results. Often the antisera agglutinated only the homologous organism; one serum (for Infant 4) agglutinated 6 different strains, 4 in group 1 and 2 in group 2.

There is considerable disagreement in the literature concerning the oxygen requirements of *Bacteroides bifidus*. The writer's experience has been that for primary isolation and for the first few transplants, strict anaerobiosis is necessary. After several transplants, aerobic growth is obtained with about 60 per cent of the strains; the remaining 40 per cent have remained obligate anaerobes, though some have been on artificial media for as long as three years.

That *Bacteroides bifidus* is the predominating organism in the feces of infants has been recognized since the work of Tissier (1900) who estimated (1908) that 85 to 90 per cent of the colonies from nurslings' stools consist of this species. What place this organism occupies in the flora of the adult is not clear from the literature. Tissier (1908) states that a normal child of five years on a mixed diet will show 70 per cent of *Bacteroides bifidus* in its stools; on a high protein diet, 50 per cent; and on a vegetable diet, 80 per cent. If this is true, the stool of the adult should contain approximately the same number. But Distaso (1912) states that *Bacteroides bifidus* is very rare in the stools of the adult, and Torrey (1918) says that this organism "is not likely to occur in large numbers in the stools of human adults except under conditions of intestinal abnormality."

In the writer's experience, *Bacteroides bifidus* (group 2) is a common inhabitant of the stool of normal adults. The actual numbers vary a great deal; some stools contain very few, others may show as high as 90 per cent of the colonies to be of this species. Of 7 individuals whose stools were cultured several times over a space of three years, 4 yielded large numbers of this organism on each occasion.

The chief biological reactions of the above 11 species of Gram-positive non-spore-bearing anaerobic bacilli are summarized in abbreviated form in table 2.

Bergey's *Manual* (1934) classifies the following Gram-positive organisms under the genus Bacteroides:

Bacteroides oviformis (Tissier, 1908). As indicated above, this species is probably identical with *Bacteroides ventriosus* (Tissier,

1908). Bergey's *Manual* states that sucrose is fermented; this is contrary to Tissier's description.

Bacteroides dimorphus, variety *longa* (Distaso, 1912). According to Distaso's description, this organism does not grow in sugar media (broth?); yet in another place he states that it grows in deep tubes of gelatin containing sugar. It acidifies and coagulates milk. Distaso's description is too meagre to enable one to recognize this organism.

TABLE 2

The chief biological reactions of the Gram-positive non-spore-bearing fecal anaerobic bacilli

SPECIES	GAS FROM GLUCOSE	INDOL	GELATIN	LEAD ACETATE	GLYCEROL	MANNITOL	SORBITOL	ADONITOL	LACTOSE	SUCROSE	TREHALOSE	GLUCOSE	MANNOSE	ARABINOSE	MELEZITOSE
B. lentus	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−
B. ventriosus	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
B. aerofaciens	+	−	−	−	−	−	−	−	+	+	+	+	+	−	−
B. biformis	+	−	−	−	−	+	−	−	±	±	+	+	+	−	−
B. tortuosus	+	−	−	−	−	+	−	−	±	+	+	+	+	−	−
B. catenaformis	−	−	−	−	−	∓	−	−	+	±	+	+	+	−	−
B. pseudoramosus	+	+	+	+	+	±	+	+	∓	−	+	+	+	−	−
B. avidus	+	−	+	+	+	−	−	+	+	+	+	+	+	−	+
B. limosus	+	−	+	−	−	+	−	+	−	∓	+	+	∓	−	−
B. cornutus	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−
B. bifidus (1)	−	−	−	−	−	∓	∓	−	+	+	+	+	+	−	−
B. bifidus (2)	−	−	−	−	−	∓	±	−	+	+	+	+	∓	+	±

Bacteroides acuminatus (Distaso, 1912). Distaso states that this is a very common organism in the stools of adults. He describes it as a short rod with rounded ends, occurring singly or in pairs. It forms acid but not gas from glucose, lactose, and sucrose. Milk is acidified and coagulated. Indol is not formed. (Bergey's *Manual* states that indol is formed.)

It seems possible that this organism is the same as that described here as *Bacteroides bifidus*, group 2, and that Distaso missed the branching forms, as other investigators have done (Cruikshank, 1925).

Bacteroides angulosus (Distaso, 1912). Distaso describes this

organism as bearing spores; it has, therefore, no place in this genus.

Bacteroides multiformis (Distaso, 1911) and *Bacteroides tenuis* (Distaso, 1911). Distaso describes these two species as spore bearers related to *Clostridium Welchii*. Le Blaye and Guggenheim (1914) also list them as spore bearers.

DISCUSSION

As stated above, the writer believes that the Gram-positive non-spore-bearing fecal anaerobic bacilli and the Gram-negative *Bacteroides* are biologically quite distinct. Reasons for this opinion were given in a previous paper (Eggerth and Gagnon, 1933); however, as the work on the Gram-positive species was at that time very incomplete, it becomes necessary to revise that list of reasons. They may now be stated as follows:

1. The Gram-positive non-spore-bearing fecal anaerobic bacilli frequently form bifid ends or lateral branches, or appear as rods with swollen central portions (Tissier's "peloton de jardinier"); or they grow in chains; or they have the aspect and arrangement of the diphtheria group. The Gram-negative *Bacteroides* are characteristically single organisms, often oval in shape, and frequently staining more heavily at the ends or around the periphery.

2. The Gram-positive species rarely ferment the pentoses, whereas the Gram-negative *Bacteroides* do this commonly. None of the Gram-positive species were found to ferment rhamnose; only 2 (*Bacteroides cornutus* and *Bacteroides bifidus*, group 2), ferment xylose; and only the latter ferments arabinose. On the other hand, 9 out of 18 species of Gram-negative *Bacteroides* ferment rhamnose; 7 ferment arabinose, and 10 ferment xylose.

3. The Gram-negative *Bacteroides* rarely ferment the higher alcohols, whereas the Gram-positive species do this much more commonly. Of the Gram-negative species, only one ferments glycerol, one ferments mannitol, 2 ferment sorbitol, and none ferment erythritol or inositol. On the other hand, 2 Gram-positive species ferment glycerol, 7 ferment mannitol, 3 ferment sorbitol, 3 ferment adonitol, 3 ferment erythritol, and occasional strains of 4 ferment inositol.

4. Of the disaccharides, lactose, sucrose, maltose, cellobiose, and trehalose, the Gram-positive species ferment trehalose *most* readily, whereas the Gram-negative species ferment this sugar *least* readily. Nine of the 11 Gram-positive species ferment trehalose; furthermore, the fermentation is usually more rapid than that of the other disaccharides. On the other hand, only 5 out of 18 Gram-negative species ferment this sugar, and those that do, often ferment it very slowly.

5. As a group, the Gram-negative *Bacteroides* are more proteolytic than the Gram-positive species. Out of 18 of the former, 12 liquefy gelatin, 9 produce indol, and 15 produce hydrogen sulfide. On the other hand, only 3 out of 11 Gram-positive species liquefy gelatin, only one produces indol, and 3 produce hydrogen sulfide.

6. The Gram-positive species usually give a flocculent growth in glucose broth, with only a slight turbidity; most of them grow poorly or not at all in peptone water without sugar. The Gram-negative *Bacteroides* usually grow diffusely in glucose broth, and grow fairly well in peptone water without sugar.

For these reasons, the Gram-positive species described in this paper should be classified separately from the Gram-negative *Bacteroides*. Some of them might be placed in the genus *Lactobacillus*; Weiss and Rettger (1934) have shown a very close relationship between *Bacteroides bifidus* and *Lactobacillus acidophilus* and there is possibly a close relationship between *Bacteroides aerofaciens* and *Lactobacillus acidophil-aerogenes*. Or the entire group might be placed in a new genus, possibly in the order *Actinomycetales*.

SUMMARY

1. One hundred and thirty strains of Gram-positive non-spore-bearing anaerobic bacilli from human feces have been studied and classified in 11 species.

2. These 11 species seem sufficiently closely related to be included in one genus. As they differ in many respects from the Gram-negative *Bacteroides*, it is suggested that they be classified either in the genus *Lactobacillus* or in a separate genus.

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A STUDY OF MICROBIC VARIATION IN A YELLOW PIGMENT-PRODUCING COCCUS[1]

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INTRODUCTION

A consideration of present views relative to the nature of microbic variation shows several differences of opinion. This study was undertaken in order to obtain further evidence in regard to the nature of variation, which for the past decade has become of interest not only to students of medical bacteriology and public health, but also to workers in the fields of agricultural and industrial bacteriology.

Microbic variation at the present time is a more or less universally accepted phenomenon. For an excellent and extensive review of the subject, including a complete survey of the earlier literature, the reader is referred to Hadley's work (1927). An extensive bibliography of the published works since Hadley's survey may be found in my original manuscript.[2]

This work was undertaken for the purpose of studying the cultural, morphological, biochemical, and to a limited degree, the immunological phases of variation in a recently isolated yellow pigment-producing coccus. The study includes a consideration of the growth cycle of this coccus which showed a motile phase. The work is limited to a discussion of the methods of obtaining the variants, the character of the changes observed, the possibility of filterable forms and a growth cycle showing changes in the morphology of the organism.

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Graduate School of the University of Minnesota, June 18, 1934.

[2] Library of the University of Minnesota, Minneapolis, Minnesota.

CHARACTERIZATION OF THE ORGANISM

The organism, a yellow pigment-producing coccus, was isolated from an apparently normal throat. When examined after 24 hours growth on an agar plate the colonies resembled *Micrococcus citreus*. However, after 48 hours the colonies became granular and in 4 to 5 days were definitely rough and showed mycelium (× 75) radiating from the periphery. Impression stains of the mycelium showed it to be made up of bundles or rows of coccus-like elements.

Morphology. The organisms were spherical, 0.8 to 1.0 μ in diameter, arranged in clusters (plate 2, fig. 1). Their morphology was studied when first isolated and again one and two years later. No marked changes were observed during that period. The organisms were Gram-positive and showed no irregular forms. Negative staining showed clear cut spherical forms in irregular clusters resembling staphylococci. The cocci were non-motile and did not form spores or capsules.

Agar plate. The streak plates were incubated at 37°C. for 24 hours and then placed in diffuse daylight at 22°C. for 24 hours. The colonies were circular, dome-shaped, 0.5 mm. in diameter, cadmium-yellow, and glistening with entire edges. The edges of the colonies became fimbriate in 4 days and later showed an abundant mycelium radiating from the periphery (fig. 2). The colonies were granular after 48 hours incubation and became definitely rough after 4 to 5 days.

Agar slant. Abundant, finely granular, cadmium-yellow; filiform, moist, entire edge (fimbriate after 4 days). The stab growth was moderate, beaded and extended to the bottom of the tube.

Nutrient broth. Moderate growth with uniform turbidity. The culture produced an abundant yellow flake-like pellicle which settled to the bottom of the tube forming a yellow viscid sediment which did not disintegrate on shaking.

Potato. Abundant, dry, yellow, spreading growth. The medium became slightly darkened.

Biochemical reactions. Acid but no gas was produced in glucose, sucrose, maltose, levulose and glycerol. Lactose, mannitol,

raffinose and salicin were not fermented. Litmus milk became acid in 3 days, but no further changes were noted after 14 days. Infundibuliform liquefaction was observed in gelatin after 7 days, becoming stratiform in 2 weeks. Indol was not formed and acetylmethylcarbinol was not produced. Moderate reduction of nitrates and marked diastatic action was shown. On amino-nitrogen assimilation medium, as used by Hucker (1924), the strain showed moderate growth and produced acid.

PURE CULTURE METHODS

The following methods were used for obtaining pure cultures of the organism: (1) serial plate method, (2) pour plate method, and (3) single cell isolation.

Twenty-four hour broth cultures were streaked on nutrient agar plates, pH 7.2. These were incubated at 37°C. and after 24 hours a well isolated colony was streaked on a second plate. After 24 hours incubation a well isolated colony from this second plate was streaked on a third plate. Colonies from this third plate were used in the experimental work.

All isolations were duplicated using pour plates. Some investigators believe that this method gives greater assurance that the colony arises from a single cell. The single cell method of Nirula (1928) was also used.

The serial plate method seems to be the best for isolating cocci in pure culture. It is reasonable to believe that a series of platings will eliminate filterable and similar forms by a process of dilution. In some cases plating was continued until all the colonies appeared uniform, and the microscopic examination of stained films from several colonies on each plate gave results that were identical.

METHODS OF OBTAINING VARIANTS

In this investigation no methods were used to induce variation other than by aging the cultures in nutrient broth, antiserum broth and broth filtrates. The usual amount of peptone (Difco), salt and meat extract was used, and the broth was adjusted to pH 7.0 to 7.2. The antiserum broth consisted of nutrient broth

to which was added 10 per cent homologous antiserum. All the media were incubated at 22°C. and 37°C. before they were inoculated.

In aging cultures duplicate flasks were inoculated at each transfer. One flask was used for the microscopic and cultural examinations each week; the duplicate was not opened until the particular experiment was completed, or until a change in the appearance of the organisms was observed in the first flask culture. By this method the chances for contamination were reduced since no flask was opened and then used for further inoculations. Furthermore, the microscopic and cultural examinations of the duplicate culture provided a control as to the results obtained from the first culture. Several sterile uninoculated broth flasks were incubated with each experiment and served as a check on the media used.

Agar streak and poured plates were inoculated in duplicate. In addition, air contaminant control plates were used when inoculating agar plates. These control plates were uncovered during the process of streaking, or pouring, and any colonies that appeared were compared with the colonies appearing on the series plates. In a number of cases control plates and plate cultures were kept for several weeks without showing signs of contamination, even though the plate cultures had been opened for the purpose of obtaining material for subculture and microscopic examination. The inoculated plates were not discarded after the initial observation, 36 to 48 hours after inoculation, but were studied over a period of 3 to 4 weeks, since it was found that interesting changes in colony structure frequently occurred after prolonged growth on agar plates. In order further to eliminate the chances of contamination, the aged broth cultures were streaked on agar (solidified in a horizontal position) in 32 oz. medicine bottles.

CHARACTERIZATION OF THE VARIANTS

Three types of variant were obtained by aging the yellow pigment-producing coccus (96) in liquid media, namely—a smooth yellow variant (96S), a rough yellow variant (96-1) and a small

rough filterable form (96FV). Intermediate variants were also observed.

Variant 96S. This smooth yellow variant occurred regularly on agar plates inoculated from antiserum broth cultures that had been aged for 14 to 60 days at 22° or 37°C. The colonies were smooth, raised, cadmium-yellow and glistening with entire edges. They were larger than the original colony (96) and unlike this type did not form mycelium. Figure 3 illustrates two smooth and two original rough colonies. The variant produced a uniform turbidity in nutrient broth, surface growth in the form of a

TABLE 1

Biochemical reactions

	ORIGINAL STRAIN	VARIANT		
		96S	96F	96FW
Glucose	+	−	+	+
Lactose	−	−	−	+
Sucrose	+	−	+	+
Maltose	+	−	+	+
Mannitol	−	−	−	+
Glycerol	+	−	+	+
Milk	+	−	+*	+
Gelatin	+	−	+	+
Diastatic action	+	+	+	+
Nitrate reduction	+	+	+	+

* Coagulated and reduced. Note: + in the case of the fermentable substances indicates acid production without gas; in the other cases it indicates a positive reaction.

ring, and a grey-white viscid sediment which readily disintegrated on shaking. When first isolated the variant consisted of a pure culture of short Gram-positive rods. After repeated transfers on agar slants during the course of a year, coccoid, bacillary and filamentous forms appeared at different periods of growth. Biochemically the variant was practically inactive, as shown in table 1. The variant was readily transferable on agar and as far as its colony structure was concerned remained stable for $2\frac{1}{2}$ years. We observed the reversion of this smooth variant to the original rough form after aging in nutrient broth for 3 months.

Variant 96-1. This rough variant was obtained by aging the original rough strain in antiserum broth for 30 days at 22° or 37°C. The colony had a wrinkled "wall" around the periphery, a somewhat wrinkled and granular center, and a mass of mycelium extending some distance from the periphery (fig. 4). The mycelium of the variant was more compact and its outward growth from the periphery was more extensive than the mycelium of the original strain.

Variant 96FV. This variant occurred on agar streak plates inoculated with nutrient broth cultures of the original organism aged at 37°C. for 21 days. The colonies appeared after the plates had been incubated for 7 days, and were visible only with the microscope. Under a Leitz ultropak (×110) the colonies had a rough "wooly" appearance and showed a mass of rhizoid mycelium extending into the medium (fig. 5). Stained films showed a mass of filaments, short and long rods, and irregular diphtheroid forms. Repeated attempts were made to subculture these colonies to nutrient agar, glucose agar and blood agar without success. The colonies were not transferable on solid media, but were transferable in nutrient broth where they produced uniform clouding with pellicle formation. These small colonies contained filterable elements and are probably identical with Hadley's (1931) G colonies. This variant will be further discussed below.

FILTERABILITY EXPERIMENTS

In this investigation a search was made for filterable forms of the yellow pigment-producing coccus, or its variants. Cultures aged in nutrient broth and in antiserum broth for 3 to 6 months were filtered through Berkefeld filters V, N and W, and through Seitz filters. The first 15 cc. of filtrate were collected in large test tubes (inside the filter flasks) containing 15 cc. of nutrient broth. The filtrates were incubated at 37°C. for 24 hours. Only 2 filtrates out of 48 were contaminated. In the filtrates in which filterable forms occurred, clouding developed after 36 hours.

A modified Hauduroy (1929) technique was used to demonstrate the presence of filterable forms. Two loopfuls of the broth filtrate were spread over the surface of nutrient agar plates.

Three plates and a bottle flask were inoculated with each filtrate. These plates and flasks and uninoculated control agar plates were incubated at 37°C. for 48 hours and then at 22°C. for 10 days. Two loopfuls of sterile broth were spread over the agar surfaces of the plates and flasks. Without flaming the loop, the broth washings were transferred to a second series of plates and flasks and incubated in the same manner as the first set. If growth did not appear on the first series of transfers a second series of washings was made from the first set of cultures. Only two series of washings were made, since it appeared that the chances for contamination increased with each series. In the successful cases growth usually appeared on the plates inoculated with the first filtrate washings. In every case where growth was detected it was checked with the bottle flask cultures and with the control plates. The original broth filtrates also were examined microscopically to detect the presence of growth.

CHARACTERIZATION OF THE VARIANTS OBTAINED BY FILTRATION

Variant 96F. Variant 96FV was inoculated into 50 cc. of nutrient broth, incubated for 10 days at 37°C. and filtered through a Berkefeld N candle. After 36 hours growth at 37°C. the filtrate gave rise to a pure culture of Gram-positive branching filaments. After cultivation on nutrient agar for 4 weeks these filaments gradually changed into short rods and finally a coccus form, morphologically and culturally similar to the original strain. The change from the coccus to the filterable form and to the original coccus as well as the R to S variation are diagrammatically illustrated in plate 1.

Streak plates made from the filtrate of 96FV after aging for 3 weeks at 37°C. showed a pure culture of small cadmium-yellow colonies (96F). These colonies grew more slowly than those of the original organism (96), were smaller and of a more uniform size. This variant had the same biochemical reactions as the original organism with the exception of its behavior in milk, in which it showed acid formation, coagulation and reduction, whereas the original strain produced only acid. The growth on agar slants, on potato medium and in nutrient broth also re-

sembled strain 96, with the exception that in broth the variant produced a ring surface growth.

Variant 96FW. This white variant appeared on agar plates inoculated from a broth filtrate of variant 96FV which had been aged at 22°C. for 3 months. The streak plates showed small yellow colonies which resembled the original strain (96). Some of these small yellow colonies, after 7 days growth at 22°C., gave rise to white daughter colonies and white sectors. Subcultures from these areas on agar remained stable for over 2 years. This white variant differed from strain 96 by fermenting lactose and mannitol.

Variant 96M. Streak plates from the broth filtrate of 96FV showed small yellow colonies. After incubation for 2 weeks at 22°C., in diffuse daylight, the colonies showed a slight dark coloration when observed through the bottom of the plates. This central area gradually became larger and after 3 weeks colonies with well developed maroon centers were seen. At the end of 4 weeks the maroon color extended to within a few millimeters of the periphery (plate 2, fig. 6). Gram-stained slides from these colonies showed no characteristic bacterial forms with the exception of a few coccoidal "shell" forms (2.0 × 0.8 μ). The staining was uneven and showed both Gram-positive and Gram-negative properties. This maroon-colored variant was transferable in broth, but thus far we have not been able to obtain the same colony form by direct transfers to agar. The maroon color was not produced in liquid media.

In addition to the filtration experiments using variant 96FV, the original coccus was aged in antiserum broth and filtered through Berkefeld and Seitz filters. After incubation at 37°C. for 10 days small pin-point colonies developed on the plates and flasks inoculated with the first filtrate washings (Hauduroy technique). Gram-stained slides from these colonies showed irregular stained forms, filaments, short and long rods, diphtheroids and a mass of faintly staining cocci. These coccus forms looked like a "shell" which had lost the material which made up the cell substance. These forms, like those obtained from the filtrate of 96FV, were not transferable on solid media. Aged

nutrient broth and antiserum broth cultures of variant 96S and 96F were also filtered. No growth was obtained on any of the plates or flasks.

GROWTH CYCLE—MORPHOLOGICAL CYCLE AND MOTILE PHASE

The original culture (96) was kept through many test tube generations and was frequently tested culturally and microscopically. After being subcultured for about 2 years on agar slants the strain spontaneously changed from cocci to rods and filaments, a change apparently associated only with the morphology. In order to determine whether this change was a part of an orderly and natural growth cycle 30- and 60-hour growth studies were made.

For the 30-hour growth study agar slants, inoculated with the coccus form, were incubated at 37°C. and examined every 3 hours in Gram-stained slides, negative stained slides and hanging drop preparations. Congo-red negative staining, according to the method of Benians (1916), was especially useful for identifying the changes in form that occurred during the morphological cycle of this organism.

During the 30-hour growth period the organism showed a complete change from cocci to rods, to filaments, to rods, to coccoidal forms to the original coccus form,—a morphological cycle completed in 30 hours. The organism changed from the coccus to a short rod in about 6 hours. Observations after 9 hours growth showed branching filaments which gradually broke up into long rods after 12 hours growth. The rods gradually became shorter and after 27 hours had changed into small coccoidal forms. These forms changed into the typical coccus, arranged in irregular clusters, in 30 hours.

For the 60-hour growth study, agar slants were inoculated with the filamentous form, which had spontaneously developed after subculturing the original coccus on agar slants for about 2 years. Examinations made every 3 hours, according to the method used in the 30-hour study, showed that the filaments gradually changed into short rods in about 27 hours. After 39 hours growth only very short rods and coccoidal forms were observed. At the end

of 60 hours the short rods and coccoidal forms had changed to cocci. This morphological change from the filamentous to the coccus form is illustrated in plate 3. It is interesting to note that it required 60 hours for the organism to change from the filaments to the cocci while it required only 30 hours to complete the cycle from cocci to filaments, to cocci.

Motile phase. During the growth study motility was observed in some of the hanging drop preparations. Further observations in nutrient broth cultures of the original organism (96) and the smooth variant (96S) showed that the short rods and coccoidal forms were actively motile. The filamentous forms showed no true motility. It was observed that motility was present only during the 24- to 48-hour growth phase, with the greatest number of motile cells and greatest activity being shown after 36 hours growth at 22°C.

In order to confirm the motility observed in the hanging drop preparations the organisms were grown in a semi-solid medium similar to that used by Jordan, Caldwell and Reiter (1934). Semi-solid agar plates were inoculated with a 10-day agar culture of the original organism (96), a 48-hour culture of the same organism which showed a short rod to coccoidal form and a 48-hour agar culture of variant 96S. The plates were incubated at 22°C. for 3 days. The semi-solid agar inoculated with the coccus form showed all non-motile colonies, the plates inoculated with the coccoidal form showed 9 motile and 15 non-motile colonies, and those inoculated with the smooth variant showed 8 motile and 2 non-motile colonies. Six semi-solid medium plates were inoculated with each strain and the results were consistent in each case.

To study further the motility of these organisms flagella-stained slides were made of the coccus, short rod form and the filamentous form of the original rough organism (96) and the short rod form of the smooth variant (96S). The flagella stain described by Safford and Fleisher (1931) and modified by Jordan, Caldwell and Reiter (1934) was used.

The flagella-stained slides confirmed previous observations on hanging drop preparations and on semi-solid medium. No flagella were observed on preparations made from the coccus form

of 96 or any of the filamentous forms while flagella were found on the short rod or coccoidal forms (10 per cent). The majority (95 per cent) of the cells of the smooth variant, which consisted entirely of coccoidal forms, showed flagella (plate 2, fig. 7). Slides made from young cultures, less than 21 hours old, or cultures over 5 days old, showed no flagella. Throughout these experiments the smooth variant showed a greater number of motile cells than the original rough organism. Colquhoun and Kirkpatrick (1932) stated that many organisms developed motility on semi-solid medium while they are non-motile when grown in the ordinary fluid or solid media. Our slides were made from agar slants and not from colonies on semi-solid medium. We are therefore certain that the motility here observed cannot be explained by growth in semi-solid medium. The flagella-stained slides verify the fact that this organism has a motile stage, the coccoidal or short rod form.

SERUM AGGLUTINATION REACTIONS

Serum agglutination experiments were carried out with the original rough organism (96) and its smooth variant (96S) in order to furnish additional evidence on the relationship between R and S forms. A study was also made of the serological relationship between these R and S forms and *Micrococcus aureus*.

Antiserum was prepared from the R and S stains. Rabbits were immunized by injecting, intravenously, 1 cc. of a saline suspension of organisms grown on agar at 37°C. for 24 hours. The rabbits were given 7 injections at 5 day intervals. They were bled from the heart 5 days after the last injection and the serum was preserved with 0.3 per cent trikresol. In the agglutination tests the antigens were prepared by suspending the growth from a 24-hour slant in 0.25 per cent saline and adjusting the density to 3 on McFarland's nephelometer. In the test, 0.5 cc. of the antigen was added to 0.5 cc. of the diluted serum and incubated in a water bath at 56°C. for 4 hours and at 22°C. for 18 hours. The results of the agglutination experiments are shown in table 2. The original rough antiserum agglutinated the homologous organism in a dilution of 1:640, but did not agglutinate the

smooth variant or *Micrococcus aureus*. The smooth antiserum agglutinated the smooth strain in a dilution of 1:1280, but did not agglutinate the original rough strain or *Micrococcus aureus*. All of the control tubes were negative. There was no antigenic relationship between the original organism (96) and the smooth variant (96S), and between these strains and *Micrococcus aureus*.

TABLE 2

Agglutination reactions

DILUTION OF ANTISERUM	ROUGH ANTISERUM			SMOOTH ANTISERUM		
	Antigen			Antigen		
	R	S	*M. aureus*	R	S	*M. aureus*
1:10	++++	−	−	−	++++	−
1:20	++++	−	−	−	++++	−
1:40	++++	−	−	−	++++	−
1:80	++++	−	−	−	++++	−
1:160	++++	−	−	−	++++	−
1:320	+++	−	−	−	+++	−
1:640	++	−	−	−	++	−
1:1280	−	−	−	−	++	−
1:2560	−	−	−	−	−	−
Control	−	−	−	−	−	−

DISCUSSION

The present studies show that a coccus goes through a progressive morphological change from cocci to short rods to long rods to filaments and back again to long rods to short rods to coccoidal forms and to cocci. Novak and Henrici (1933) reported similar morphological changes in a yellow staphylococcus isolated from a filtrate of a broth culture of an actinomycete. The colony structure and cultural characteristics of their organism are essentially similar to the coccus described in this paper.

Other workers have reported changes in bacterial forms which simulate a morphological cycle. Wittern (1933) working with "*Microbacterium*" *mesentericus* found that rods and filaments divided into coccus-like fragments which developed into the normal form in liquid medium. Cunningham (1930–1931) observed that *Bacillus saccharobutyricus* (von Klecki) may produce coccoid,

rod, dwarf growth and fungoid forms corresponding to the types of Löhnis and Smith. Henrici (1928) observed a series of morphological changes in various bacterial species to which he applied the term "cytomorphosis."

Mellon's (1919) study on a fuso-spirillary organism and his several other studies on microbic heredity display a similarity to our observations. He showed that the fuso-spirillary organism could produce various forms which showed transformations from one form to another; transformations between staphylococci and branching filaments and between diphtheroid forms and branching filaments. In 1927 he presented bacteriologic evidence for an *in vivo* life cycle that involved bacillary coccus transformations. The report shows a relationship between pigmented staphylococci and the long thread and bacillary forms that apparently dissociated *in vivo*. In another study Mellon and Jost (1926) showed that *Bacillus alkaligines* can exist as a diplococcus and then be transformed into pure line bacillary and filamentous forms, variants which bred true and were serologically reciprocal.

We found no reference in the literature to a growth cycle transformation between various phases which differ not only in morphology, but also physiologically. The coccoidal and short bacillary forms showed motility and flagella formation, a characteristic not possessed by either the filaments or the true cocci.

In our work we present a pleomorphic cycle which forecasts the direction into which the culture can dissociate. Two of the forms, a filamentous and a short rod (S form) were stabilized and later reverted to the original coccus form. Mellon and Jost (1926) have shown that the colon-alkaligenes group may exist in a stabilized actinomycotic stage. Our growth cycle meets the requirements of Mellon's[3] pleomorphic principle, "Each characteristic pleomorphic phase of a microörganism is capable of being stabilized as such, the culture thereafter being capable of multiplying into what were formerly pleomorphic forms." Mellon (1926) pointed out that the various phases of the pleomorphic cycle represent the ontogeny of the strain; when the phases are

[3] Personal communication.

stabilized through mutation they collectively represent the phylogeny of the strain. Winslow (1935) stated, "If a unicellular organism shows a definite series of morphological and physiological alterations in response to certain changes in environment which are likely to occur with reasonable frequency in its natural life we may call it a 'life cycle' if we wish. . . .".

Mellon (1927) found that bacteria may reproduce by various methods besides transverse fission. He reported that transitions of a major type, such as between filaments and cocci and *vice versa* were brought about through the medium of reproductive forms, either filterable microgonidia or macrogonidia. Some of our observations indicate that the coccus reproduced by other methods than transverse fission. We observed bud-like structures on some of the filaments, on negative-stained slides, and deeply staining bodies in the diphtheroid forms of the G colony which resembled gonidia.

The various rough and smooth colonies were distinct types and reproducible, true to form, many times under varying conditions. Arkwright (1921) found that the differences between the various colony types were often indefinite. Two of our variants were of an unusual type, one with a pronounced "wall"' structure around the periphery, the other a maroon-colored colony. Fleck and Elster (1932) in studying the variation of streptococci also obtained a rough colony with a "wall" around the periphery and a rust-colored variant.

The elements contained in the G colony were filterable and gave rise to filamentous forms which developed into the original coccus. Our organism passed through an R, S and G form similar to the cyclic change described by Hadley (1931) in his studies on the life history of the Shiga dysentery bacillus.

In evaluating any work of this nature it is necessary to show, within reasonable limits, that the observations made cannot be explained by contamination. The results reported here are reproducible, and the majority of forms were obtained from various sources. It may seem that some of our observed variations appear to be tinted with an extreme view of pleomorphism, but in spite of this, all the variants represent a phase in the life cycle

of the original organism. We therefore feel that the common argument of contamination cannot be invoked to explain these findings.

SUMMARY

Microbic variation was studied in a recently isolated strain of an unidentified yellow pigment-producing coccus. Variants were produced by growing the organism in nutrient broth, 10 per cent antiserum broth and in broth filtrates. After aging the cultures in nutrient broth and antiserum broth three different variants were observed; a smooth yellow variant, a rough yellow variant and a filterable form. Several intermediate forms were also obtained.

Variants were obtained from the broth filtrates of the filterable form. After incubation for 36 hours a filamentous form was obtained which developed into the original coccus after prolonged growth on agar. A white variant and a maroon-colored variant were also obtained from aged broth filtrates of the filterable form.

No antigenic relationship was found between this yellow pigment-producing coccus and *Micrococcus aureus*, or between the the smooth variant and *Micrococcus aureus*. No cross agglutination occurred between the rough type and the smooth variant.

The original strain after cultivation on nutrient agar for two years spontaneously changed from a coccus to a filamentous form. Growth studies showed that this filamentous form was part of a morphological cycle which was completed in 30 hours. During this cycle the cocci changed to rods and filaments which finally reverted to cocci. The filamentous form required 60 hours to go through the various forms from filaments to cocci.

During this morphological cycle motility was shown in the short rod or coccoidal phase. This motility was observed in hanging drop preparations, by the presence of motile colonies in semi-solid medium and by flagella stained slides.

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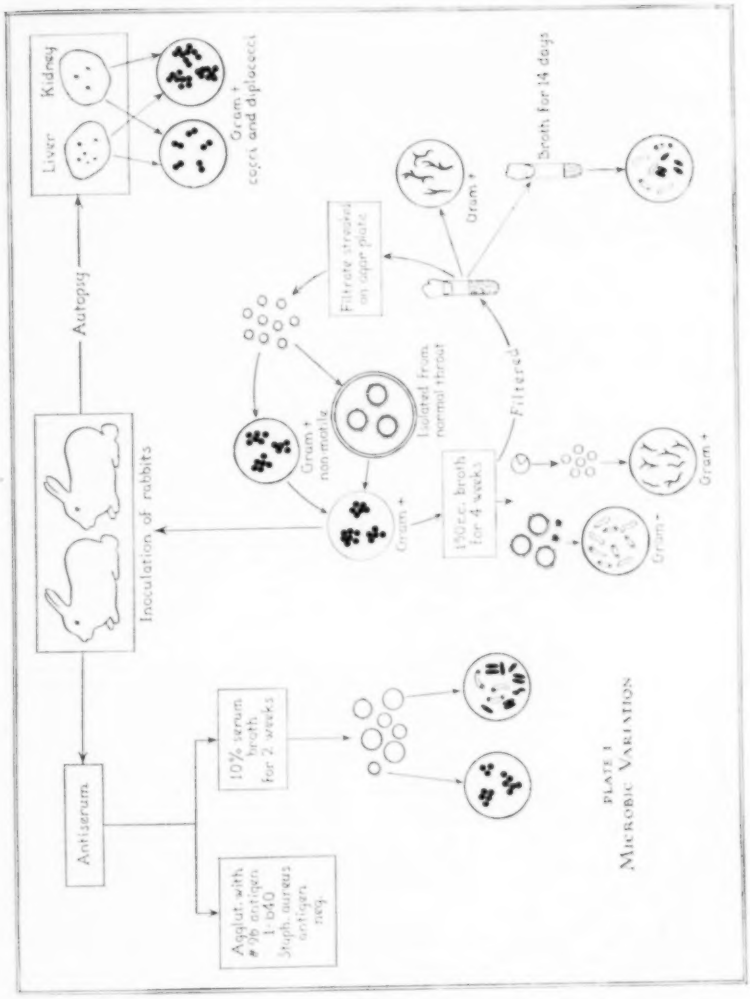

PLATE 1
MICROBIC VARIATION

(Francis E. Colien: Microbic variation in pigment-producing coccus)

UNIV OF MICH.

PLATE 2

FIG. 1. Unidentified yellow pigment-producing coccus, original rough, from twenty-four hour agar slant, stained by Gram's method. Gram-positive. × 1000.

FIG. 2. Original rough colony of the yellow pigment-producing coccus, 6 days growth on nutrient agar. Note the mycelium extending from the periphery. Transmitted light. × 75.

FIG. 3. Two smooth colonies of variant 96S, and two original rough colonies, 10 days growth on nutrient agar. Transmitted light. × 50.

FIG. 4. Rough variant colony 96-1 obtained by aging the original strain in 10 per cent antiserum broth, 14 days growth on nutrient agar. × 16.

FIG. 5. A small colony, filterable form, 7 days growth on nutrient agar. Leitz ultropak microscope. × 110.

FIG. 6. Variant colony 96M, maroon center, 4 weeks growth on nutrient agar. × 16.

FIG. 7. Safford and Fleisher flagella stain of the short rod or coccoidal form of the yellow pigment-producing coccus from a twenty-four hour agar slant. × 1800.

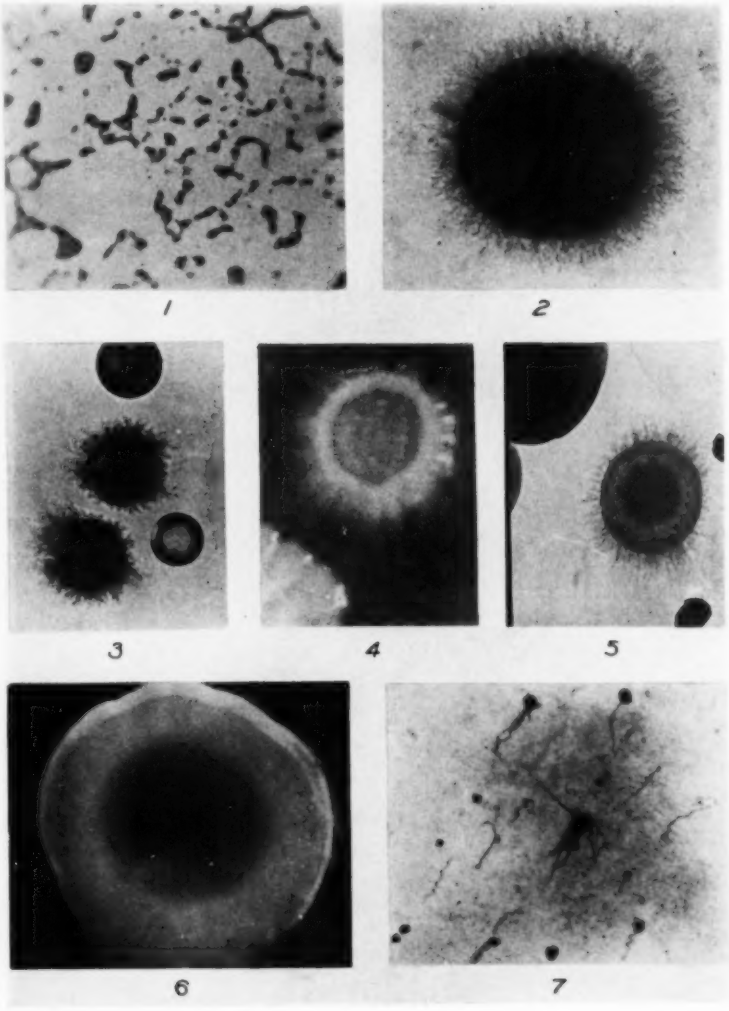

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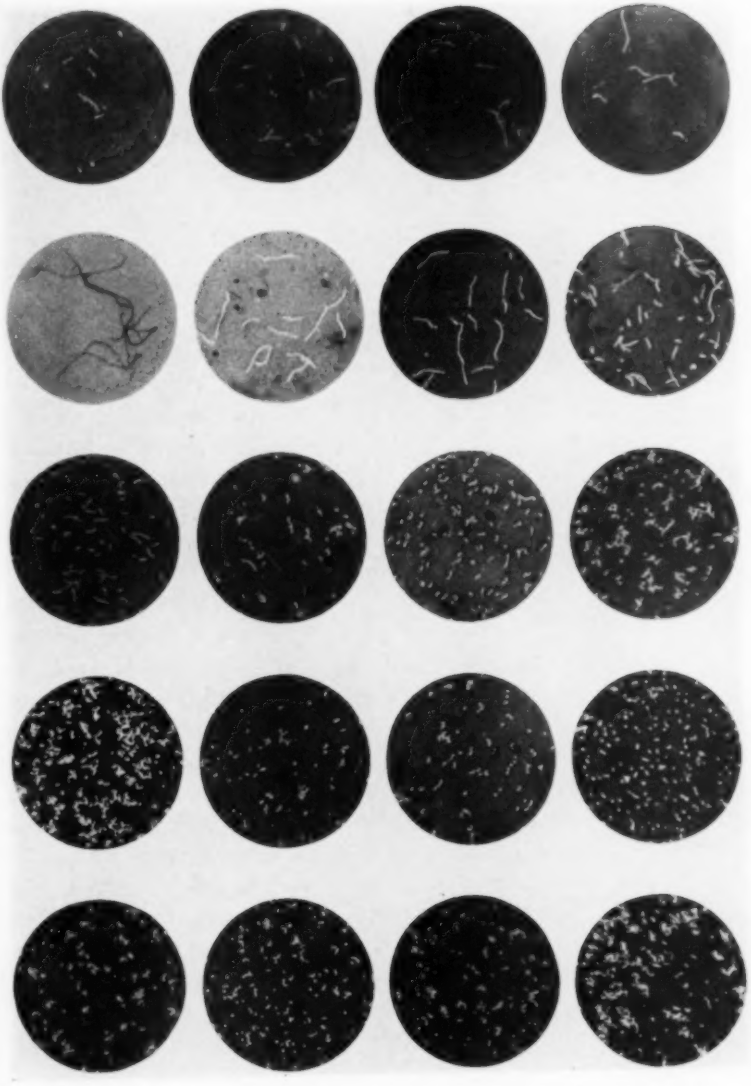
PLATE 3

MORPHOLOGICAL VARIATION OF A YELLOW PIGMENT-PRODUCING COCCUS. THE TRANSFORMATION OF FILAMENTS TO COCCI

Each figure represents three hours in the growth cycle; 3, 6, 9, up to 60 hours at 37°C. Negative stained preparations (Congo red) from nutrient agar. × 1000.



(Francis E. Colien: Microbic variation in pigment-producing cocens)

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THE OCCURRENCE OF A STRAIN OF AZOTOBACTER CHROOCOCCUM WHICH DOES NOT FERMENT MANNITOL

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INTRODUCTION

Azotobacter is widely distributed in soils having a pH value of 6.0 or above.[1] For detecting its presence, various methods are used, such as plating with Ashby's mannitol agar, scattering soil over the surface of an Ashby's agar plate or over the surface of a silica gel plate to which mineral nutrients and mannitol have been added, and the inoculation of Ashby's solution with soil and observing the growth of the *Azotobacter* film. Almost without exception, media for culturing *Azotobacter* have contained mannitol, in spite of the fact that this bacterium is known to use a variety of carbohydrates, organic acid salts and other alcohols as sources of energy.

Skinner (1929), finding it difficult to distinguish between the white *Azotobacter chroococcum* and other soil bacteria on Ashby's medium, suggested that dextrin be substituted for the mannitol in that medium in order to increase the pigmentation. He used this medium modified by the addition of nitrate for plating out enrichment cultures of *Azotobacter* which had been grown, however, in a mannitol solution.

By the use of dextrin Ashby's agar the writer (1934) obtained from a Colorado soil a culture of *Azotobacter chroococcum* which differed from the usual type only in its inability to use mannitol

[1] For a review of the literature on the distribution of *Azotobacter* and for the constitution of Ashby's medium, see Waksman's "Principles of Soil Microbiology," 2nd Edition, 1932.

as a source of energy. It is the purpose of this paper to show the frequency with which this mannitol-negative strain occurs in soils from widely separated regions.

METHODS AND MATERIALS

About eighty soil samples representing various parts of this and of foreign countries were plated on dextrin, starch, or sucrose nitrogen-free agar using dilutions of 1:10 and 1:100. In some cases, 0.1 gram of soil was scattered over the surface of a cooled agar plate instead of using dilutions. After an incubation of a week at 28°C. colonies resembling *Azotobacter* were picked off and purified on sucrose-Ashby agar. Cultures identified microscopically as *Azotobacter* were then transferred to starch-, dextrin-, mannitol-, and glycerol-Ashby's agar, and observations made at intervals as to the character of the growth.

RESULTS

Of the soils tested, the 8 samples of soil from the Red River Valley in North Dakota contained, as a whole, the greatest percentage of the mannitol-negative strain of *Azotobacter chroococcum*. In 4 cases, (nos. 53, 259, 260, and 263, table 1), all the cultures isolated failed to utilize mannitol, whereas in only 2 cases (nos. 72 and 92), did the mannitol-positive strain predominate. The pH value of the soil, as determined by the quinhydrone electrode or colorimetrically, seems to bear no relationship to the presence of the mannitol-negative strain.

In contrast to the above samples from one locality in North Dakota, 9 samples of soil from widely separated regions in Utah were plated out. In order to save space, the results are summarized in table 2. It should be explained, however, that in one sample the ratio of the mannitol-negative to the mannitol-positive strain was 37:1, and in another, 15:7. In 4 samples the ratio was about even whereas in the remaining 3 samples, only the mannitol-positive strain was present.

The 3 soil samples from Colorado came from the same general locality. One contained 10 mannitol-negative cultures to 1 culture fermenting mannitol; in another the ratio was 3:7. The

third sample contained only those using mannitol which made the average of the 2 strains slightly in favor of the mannitol-positive strain (table 2).

TABLE 1

Showing the relative numbers of mannitol-negative and mannitol-positive cultures isolated from North Dakota soils and the pH value of the soil samples

SAMPLE NUMBER	NUMBER MANNITOL-NEGATIVE CULTURES	NUMBER MANNITOL-POSITIVE CULTURES	pH VALUE OF SOIL
53	10	0	7.8
72	1	9	7.6
92	2	10	8.0
153	6	3	7.6
165	9	2	7.0
259	12	0	7.6
260	13	0	7.6
263	10	0	7.8
Total	63	24	

TABLE 2

A summary of the mannitol-negative and the mannitol-positive cultures of Azotobacter chroococcum isolated from soils of various localities

LOCALITY	TOTAL SOIL SAMPLES	NUMBER MANNITOL-NEGATIVE CULTURES	NUMBER MANNITOL-POSITIVE CULTURES
Utah	9	73	56
Colorado	3	13	15
Nebraska	4	9	22
Texas	1	3	4
Louisiana	7	0	59
Washington, D. C.	2	0	34
Delaware	7	9	57
Pennsylvania	1	0	12
Hawaii	4	5	42
Chile	4	3	41
Greece	1	5	5
Turkey	2	0	24

In the four samples from the Scottsbluff region of Nebraska, a smaller proportion of the mannitol-negative strain was found, only 1 sample showing a predominance of the negative strain

(6 to 3). In another the ratio was 3:6, whereas in the remaining 2 samples, no mannitol-negative cultures were found. In the sample of Texas soil, the 2 strains were about even.

The 7 soil samples from Jeanerette, Louisiana, were all taken from the same experimental field. No mannitol-negative cultures were found. These results and those obtained on the 2 soils from Washington, D.C., and the one soil from Port Royal, Pennsylvania, show that the demonstration of the presence of the mannitol-negative strain is not due to technique nor to personal factors. A total of 105 isolations from these 3 soils were made without a mannitol-negative strain being found.

In the 7 soil samples from Delaware, taken from various plots of the same experimental field, there was a fairly uniform occurrence of the mannitol-negative cultures, averaging about 15 per cent.

Of the cultures isolated on starch agar from the 4 Hawaiian soils, about 10 per cent failed to grow on mannitol agar. From Chile, only 1 of the 4 samples showed the presence of the mannitol-negative strain, the other 3 samples lacking it entirely. Half of the cultures isolated from the soil sample from Greece were the mannitol-negative strain, whereas no negative cultures were obtained from the 2 soil samples from Turkey.

DISCUSSION

The question naturally arises whether the mannitol-negative strain should be called a new species. The author has preferred to designate this particular bacterium merely as a strain of the common form for the following reasons. The character of the growth on Ashby-base agar containing glucose, sucrose, starch and dextrin, in general, was the same whether mannitol-positive or mannitol-negative strains were used. For more detailed study, 19 cultures of the mannitol-negative strain and 28 cultures of the positive strain were used for inoculating nutrient agar, milk and soybean agar. The poor growth or absence of growth on these media was common to both strains, as was, also, the ability or the inability to use glycerol. In a few cases, the mannitol-posi-

tive cultures seemed to use starch poorly, but this was not consistent. Neither was there any consistent difference in the amount of nitrogen fixed, nor in the microscopical appearance of the cells.

Whether the predominance of the mannitol-negative strain in certain soils of North Dakota, Utah and Colorado is significant can not be said from the data at hand. The fact that some soils contain only this strain makes it imperative to use a carbohydrate medium for demonstrating or isolating *Azotobacter* in those regions. Since the data have shown a wide distribution of the mannitol-negative strain, it is obvious that it would be dangerous to rely upon mannitol in any case to give an accurate picture of the *Azotobacter* flora.

The pH value of the soil did not seem to be correlated with the presence of the mannitol-negative strain in any group of samples.

During the course of this work an article by Kluyver and van Reenen (1933) appeared in which it is stated that *Azotobacter agilis* isolated by them from canal and ditch water differs from *Azotobacter Vinelandii* in that it does not ferment mannitol. This may mean that the utilization of mannitol by the *vinelandii-agilis* group is as variable as that found in the *chroococcum* group. Unfortunately the writer has been unable to isolate an *Azotobacter* producing the green-fluorescent pigment of *Azotobacter Vinelandii* and *Azotobacter agilis*, although several hundreds of platings have been made over a period of about twenty years from soils of this country and Germany in addition to those reported in this paper. On the other hand, *Azotobacter chroococcum*, the black and the white types, is practically ubiquitous providing the soil reaction is favorable. This has led to the general use of the term *Azotobacter* as synonymous with *Azotobacter chroococcum*.

SUMMARY

A strain of *Azotobacter chroococcum* which could not use mannitol as a source of energy was isolated from various soils by substituting a carbohydrate such as dextrin, starch or sucrose for mannitol in Ashby's agar. It has been designated as a man-

nitol-negative strain, since the only distinguishing characteristic seems to be its inability to utilize mannitol.

In certain soil samples from North Dakota, Utah and Colorado this mannitol-negative strain was the only *Azotobacter* present, whereas in other soil samples from these same states, its number varied from 0 to 70 per cent of the *Azotobacter* population.

The mannitol-negative strain was also found in varying proportions in soils from Nebraska, Texas, Delaware, Hawaii, Chile and Greece.

This strain was not found in soil from Washington, D. C., Jeanerette, Louisiana, Port Royal, Pennsylvania, and Turkey.

CONCLUSION

Since a strain of *Azotobacter chroococcum* which can not use mannitol has been found to be widely distributed in soil, it is concluded that a carbohydrate (sucrose, dextrin or starch) should be substituted for mannitol in Ashby's medium unless tests have shown that the *Azotobacter* flora present in that particular soil sample is capable of utilizing mannitol.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH, MINNEAPOLIS, MINNESOTA

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PROBLEMS IN NOMENCLATURE AND CLASSIFICATION NOW BEFORE THE INTERNATIONAL COMMITTEE ON BACTERIOLOGICAL NOMENCLATURE. *Robert S. Breed*, New York Agricultural Experiment Station.

The first International Congress for Microbiology held at Paris in 1930 authorized the organization of a Nomenclature Committee for the International Society for Microbiology, with two permanent secretaries; one primarily to represent medical and veterinary bacteriology, and the other primarily to represent other phases of bacteriology. Dr. R. St. John-Brooks of the Lister Institute and the author of this abstract were selected as these permanent secretaries. Nineteen countries have responded to the invitation to appoint representatives on this Nomenclature Committee. This committee was also instructed to coöperate with a Subcommittee on Bacteriological Nomenclature, appointed by the authorities in charge of the International Botanical Congresses.

The first proposal submitted to the Nomenclature Committee was drawn up by Dr. R. E. Buchanan and had for its object the conservation of *Bacillus* as a bacterial generic name, the fixing of the type species, and of the type or standard culture.

A sub-committee was also appointed to consider the classification of organisms belonging to the genus *Salmonella*.

Problems in nomenclature should be presented to this International Nomenclature Committee for review as they arise. The second International Congress for Microbiology is to be held in London, July 27–August 1, 1936.

STUDIES ON EXPERIMENTAL POLIOMYELITIS AND ENCEPHALITIS. *Edward C. Rosenow*, Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minnesota.

By the use of improved methods, especially by cultivating streptococci in a new medium, autoclaved chick mash, I am now succeeding in producing with considerable regularity the symptoms and lesions of poliomyelitis and of encephalitis in animals following intracerebral and other injections of the respective streptococci thus grown, and far removed from virus, and of filtrates of old cultures. By transferring to other animals the emulsions in sodium chloride solution, or filtrates of the emulsions of the brain and spinal cord of these animals, characteristic symptoms and lesions in turn develop as in the case of studies on "natural" virus. On further transfers, as many as twenty-six thus far, the experimentally produced viruses became thoroughly established and behave quite like the "natural" viruses of these diseases.

Each of four strains have been passed consecutively through the "strepto-

coccal to virus" and through the "virus to streptococcal" phase three times.

Symptoms and lesions of poliomyelitis or encephalitis have thus far been produced with "viruses" derived from these four strains of streptococci in nine, nineteen, nine, and six monkeys, respectively, or forty-three in all, and in large numbers of mice, guinea pigs and rabbits.

CONCERNING THE ALLEGED FIXATION OF NITROGEN BY GERMINATING PEAS. *Elizabeth M. Smyth and P. W. Wilson*, University of Wisconsin.

Nerina Vita and collaborators at the University of Bologna, Italy, have published experiments which apparently demonstrate fixation of atmospheric nitrogen non-symbiotically by seeds of peas, lupines and horse-beans when germinated in presence of certain stimulants. In view of the importance of the results, confirmation or denial of the claims by investigation in other laboratories is desirable.

The results of numerous experiments by the authors, with peas and under conditions which Vita claims are necessary for the fixation of atmospheric nitrogen, may be summarized as follows:

1. Increases in the nitrogen content of peas as measured by the Kjeldahl method are readily observed when the seeds are germinated in the absence of bacteria. The apparent gains often reach 10 to 15 per cent of the total nitrogen present.

2. The observed change in nitrogen content occurs in the presence of distilled water alone, hence the stimulating materials used—alkaloids, salts, glucose—are not necessary.

3. Evidence is offered which indicates that the apparent gain in nitrogen probably arises from inadequacies of the Kjeldahl method for nitrogen analyses in this type of experiment, viz., one in which complex nitrogenous compounds are undergoing changes. Because of the inaccuracies of this method, it is proposed that uptake of free nitrogen by gasometric means be a required demonstration before proof of biological fixation is established.

THE EFFECT OF CHANCE ON THE MORTALITY OF EXPERIMENTALLY INFECTED ANIMALS. *H. O. Halvorson*, University of Minnesota.

In a mathematical consideration of the effect of chance in the experimental infection of animals, it was found that one of two assumptions had to be made in order to get a solution to the problem. First, we may assume that a critical number of organisms (the number contained in one infective dose) must be injected before infection sets in. This is analogous to stating that the probability of an organism's growing in the animal is unity when an infective dose or more is injected, whereas the probability of an organism's growing in the animal is zero if less than an infective dose is injected. Second, we may assume, instead, that the probability of an organism growing in the animal is independent of the number of organisms injected, and that this probability has a real value, usually less than 1. It becomes equal to 1 only in the limiting case where the resistance of the animal is very low and the virulence of the organism very high.

If the first assumption is operative, it can be shown that chance is relatively unimportant in experimental infection, except in the limiting case where only one organism is required to produce an infection. If the second assumption is operative, it can be shown that the effect of chance is important in all cases and is independent

of the resistance of the animal and the virulence of the organism.

ON THE NATURE OF FILTERABLE VIRUSES. *Robert G. Green*, University of Minnesota.

Filterable viruses are best considered as incomplete forms of life developed by retrograde evolution from pathogenic microörganisms of visible size. As the tapeworm has lost both functional and anatomical digestive system by intestinal parasitization, so have intracellular microbes lost cellular function and substance by intraprotoplasmic existence. Living within protoplasm offers such great opportunity for loss of function with replacement from the metabolism of the host cell that the usual result of intracellular adaptation is the evolution of a highly incomplete individual. Such retrograde simplification may proceed to an extreme point where little remains of the original intracellular microbe but the substance and vital processes necessary for the reproduction of its kind. Functionally filterable viruses are complete forms of life when within a living cell where their own limited vital activities are supplemented by the metabolic processes of the host cell. Most filterable viruses have probably originated from the sporozoan group of protozoa and the various groups of pathogenic bacteria, which accounts for the great diversity of the ultramicrobes.

A COMPARISON OF LIQUID MEDIA FOR THE QUANTITATIVE ENUMERATION OF ESCHERICHIA COLI BY THE DILUTION METHOD. *C. E. Skinner*, University of Minnesota.

By means of the dilution method and the tables of Halvorson and Ziegler, "counts" of *Escherichia coli* were made on 100 samples of human feces using (1) standard lactose broth, (2) the alkaline lactose broth of Janzig and Montank, (3) Perry's modified Eijkman test, and (4) formate-ricinoleate-lactose broth. Comparing media (1) and (2), there was the variability expected by the laws of chance. Comparing media (1) and (3), there were more *E. coli* by the lactose broth method but the difference was not large and possibly not significant. Comparing media (1) and (4), there was a significant number of *E. coli* repressed in the formate-ricinoleate media. About one-third of the samples were confirmed by standard methods. Media (1) and (2) invariably produced *E. coli*. Occasionally from medium (3) and frequently from medium (4) only glucose-fermenting aerobes were recovered.

A GROWTH FACTOR FOR RHIZOBIA. *D. W. Thorne and R. H. Walker*, Iowa State College.

A study of the nutritional requirements of the root-nodule bacteria has demonstrated the necessity of an accessory factor for their growth. Two species of *Rhizobium* were studied. They were not able to maintain growth when continuously cultivated in a synthetic medium containing only C.P. chemicals, including mineral salts, a nitrogen source of KNO_3, and sucrose. Upon replacing the C.P. sucrose with commercial cane sugar the organisms were able to grow. Presumably cane sugar contains a factor necessary for the growth of rhizobia. A concentrated preparation of the growth substance was prepared by extracting cane sugar with absolute alcohol. Yeast extract contains sufficient of the factor to initiate maximum growth of the organisms. The stimulative factor in yeast extract was concentrated by alcoholic extraction. These results con-

firm the report of Allison and Hoover of an accessory factor for *Rhizobium*.

Asparagin, aspartic acid and related compounds were not able to replace the growth factor, but acted as very readily available sources of nitrogen. The organisms were able to attack the amino group of asparagin with greater ease than the amid group. The two carboxyl groups increased the availability of the amino group and also promoted the growth of the organisms.

PRELIMINARY INVESTIGATION OF THE OCCURRENCE AND DISTRIBUTION OF AZOTOBACTER IN IOWA SOILS. *William P. Martin and R. H. Walker*, Iowa State College.

Two hundred sixty-eight Iowa soils have thus far been examined for the presence of *Azotobacter* by means of the Winogradsky spontaneous-culture and the Curie agar-plate-methods. These 268 samples include 54 soil types and 37 soil series and have been collected at random from the five soil areas of the state—Wisconsin Drift, Iowan Drift, Missouri Loess, Southern Iowa Loess, and Mississippi Loess.

Of the 268 soils examined, 118, or less than half, have reacted positively. In general the Loessal and Iowan Drift soils have reacted negatively. The Webster soils and to a lesser extent the Clarion soils of the Wisconsin Drift have reacted positively. The Terrace soils are quite generally positive. The best growth obtained occurred on the Bottomland soils which were practically all positive.

The presence of the *Azotobacter* in Iowa soils was correlated with the hydrogen-ion activity. The limiting pH value was 6.00. The best growth occurred in soils with a pH of 7.5 or above.

The factors affecting the occurrence and distribution of *Azotobacter* in soils were briefly discussed.

THE UTILIZATION OF CO_2 BY THE PROPIONIC ACID BACTERIA IN THE DISSIMILATION OF GLYCEROL. *H. G. Wood and C. H. Werkman*, Department of Bacteriology Iowa State College, Ames.

It has been established with several species of *Propionibacterium* that total CO_2 liberated during fermentation of glycerol plus that remaining in the form of carbonate is less than the original CO_2 added as carbonate. This decrease is believed to result from utilization of CO_2 by the bacteria during their dissimilation of glycerol. Carbon and oxidation-reduction balances support this view.

The 4- and 2-carbon cleavage of the glucose molecule to succinic acid and acetaldehyde, as proposed by Virtanen and supported by Wood and Werkman, requires further substantiation. The formation of succinic acid does not necessitate an evolution of CO_2 as formerly proposed, even though the succinic acid arises from a condensation of acetic acid, because the CO_2 may be utilized as rapidly as formed. The formation of succinic acid from glycerol involves a synthesis. This may possibly be interpreted in favor of the theory that succinic acid is formed by a condensation of two molecules of acetic acid. The 4- and 2-carbon cleavage must account for one molecule of a 2-carbon compound for each molecule of succinic acid formed, and there was only a small quantity of 2-carbon compounds produced. Even though a 4- and 2-carbon cleavage occurs it is still necessary to assume a 2-carbon synthesis to get rid of the 2-carbon compound.

A STUDY OF METHODS USED FOR THE ISOLATION OF BRUCELLA ABORTUS FROM MILK. *C. P. Fitch and Lucille M. Bishop*, University of Minnesota. Twenty-four samples of milk natur-

ally infected with *Brucella abortus* were studied. Guinea pigs were inoculated intraperitoneally with eight types of inoculum taken from each of the above samples. Cream, milk and sediment obtained by gravity separation and centrifugalization, whole milk and mixtures of sediment and cream were inoculated. The guinea pigs were autopsied four weeks later. The spleens were cultured and the cultures were incubated under 10 per cent CO_2. An agglutination test was made with the blood serum obtained from each. The milk sample was considered positive only if cultures of *Brucella abortus* were isolated from the spleen. A larger number of positive results were obtained when cream was inoculated in some form. The skim milk layer seldom contained sufficient numbers of bacteria to infect a guinea pig. Sediment inoculation gave fewer positive results than those of the cream layer. The use of gravity cream for guinea pig inoculations and cultures appears to be the method of choice. The numbers of positive results were greater and the collection of cream by this method is extremely simple.

Raw milk was shown to have a definite growth-inhibitory action on cultures of *Brucella abortus*. The action corresponded to the so-called "bactericidal action of milk." This action may explain the failures of direct cultures from cream which is positive by guinea pig inoculations.

INSECTS AS CARRIERS OF CLOSTRIDIUM BOTULINUM. *M. F. Gunderson*, University of Minnesota.

The part played by biotic factors in epizoötic avian botulism (duck sickness) was discussed, and it was shown that various forms of aquatic life contribute to the toxin-producing substrate.

In describing the epizoötic in the Least Sandpiper, the hitherto unknown (in the United States) life cycle of an hydrophylid water beetle was considered. Large areas of Tule Lake (California) are covered by a mat of *Cladophora*. The surface of this algal mat is dotted with large numbers of cocoons which have been formed by the hydrophylid water beetle by felting together masses of *Cladophora* filaments. The epizoötic among sandpipers occurred when the hydrophylids were in the larval stage, and lasted for a week or ten days. Large numbers of hydrophylid larvae were found in the stomachs of both sick and well sandpipers. *Clostridium botulinum*, type C, was demonstrated in cultures made from larvae and also from the cocoons. When the insects were in the pupal and adult stages and were no longer delectable or available, sandpipers did not represent any great portion of the affected bird population. The moist algal basket containing dead and putrefying larvae undoubtedly constituted the source of toxin for sandpipers.

This insect has been identified as *Enochrus Hamiltoni* Horn. Observations regarding hydrophylids which build cocoons of algae in this manner have been made by Wesenberg-Lund in Denmark, although the insect was not completely identified.

A PRECISION PHOTOMETER FOR THE STUDY OF SUSPENSIONS OF BACTERIA AND OTHER MICROÖRGANISMS

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One of the most characteristic properties of suspensions of bacteria, yeasts, and other microörganisms, is turbidity. As this turbidity will evidently vary as some function of the number of organisms per unit volume, there have been many attempts to substitute the observation of turbidity for the laborious and time-consuming methods of direct and of plate counting. Simple methods, involving matching a tube containing the experimental culture against a series of standard suspensions prepared by dilution, have been widely employed for the purpose of growing cultures to a standard concentration, and for measuring crops. Descriptions of various techniques may be found in Dreyer and Gardner (1916), Heckscher (1921), Hotchkiss (1923), Peskett (1927), Krueger (1930), and Andresen (1932).

In general it may be said that these methods have not proved acceptable as substitutes for either plate or direct counting except where speed was more essential than accuracy. This is in part due to the fact that the error in matching tubes is probably at best of the order of plus or minus 5 per cent, and may easily be much greater, and there are also possibilities of systematic errors of considerable magnitude being introduced in the preparation of the standards, or through their deterioration. In addition, there did not seem to be any very consistent correlation between turbidity and plate or total counts under varying growth conditions.

[1] This paper was prepared for publication while at the Department of Public Health, Yale Medical School, with the aid of a grant from the Rockefeller Foundation.

During recent years there has been a rapidly increasing use of photometers in an effort to increase the accuracy of the determination of turbidity. In some cases, as will be seen below, there has been an attempt to interpret these more accurate turbidity determinations in terms of differences in the suspended bacteria other than increase in number. In other investigations, where the principal interest has been in the determination of number, attempts have been made to increase speed and accuracy by the employment of thermocouples or photoelectric cells to eliminate the subjectivity of visual methods. While these instruments have varied widely in design they all belong to one of two optically distinct types; those in which the turbidity is estimated from the intensity of the light scattered by the experimental suspension, and those in which the transmission of a beam of light by the suspension is measured. The terms "turbidimeter" and "nephelometer" have been so indiscriminately applied to both types that they have today little more than general significance. In order to avoid confusion, instruments of the first type, which measure something at least analogous to the Tyndall effect, will here be called "tyndallmeters." For the second type a considerable number of names could be employed, but they will here be referred to as "densitometers." This name is in common use in the literature of photometry and arises from the practice of expressing the transmission data as "optical densities." This term will be defined below. The distinction between these two types will perhaps be clearer if we consider briefly the fate of a collimated beam of light incident normally on a suspension of cells contained in a parallel walled glass cuvette (of the type commonly used for absorption measurements or for liquid filters).

Let us assume that the incident collimated beam has a constant flux[2] (P_o), and that when we place in its path the glass

[2] It has seemed preferable to discuss the distribution of the incident beam in terms of flux (P) rather than the more commonly used intensity (I). Flux (energy per second) is an unambiguous quantity. The term intensity is, strictly speaking, only applicable to divergent light and is defined as "flux per unit solid angle," while what has been called the "beam intensity" of collimated light is "flux per cm^2." Both types of intensity occur in this problem, and there would seem to be no object in introducing this additional complication as with objective instru-

cuvette, filled with the suspension medium alone, a small amount of energy is removed from the beam by reflection at the various air-glass-liquid interfaces, and by absorption or scattering in the glass or the medium. The remaining flux (P_m) will be considered as being transmitted undeviated. If we now introduce into the cuvette a suspension of cells, a certain amount of flux (P_c) will come into contact with the suspended cells. This will in part be removed from the beam by absorption (P_a), and the balance transmitted or reflected by the cells as the scattered flux (P_s). The residual flux (P_u) will be transmitted without contact with cells and therefore without deviation.

From the previous definitions it will be seen that tyndallmeters are concerned only with the measurement of some portion of the flux P_s, or with the comparison of its intensity in some given direction, usually 90° to the incident beam, with a corresponding intensity derived from a similarly illuminated standard suspension. Densitometers, on the other hand, are designed for the measurement of P_m, and of a second experimentally determinable quantity, the flux P. This flux will include P_u and the fraction of P_s which is transmitted by the suspension within such angles as to permit it to reach the photosensitive element of the instrument. The optical density (D), as determined by any given instrument, being defined as the common logarithm of the ratio of these two measurable quantities,[3] we may write:

$$D = \log (P_m/P) \tag{1}$$

The only tyndallmeter which seems to have been employed at

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ments the quantity actually measured is the flux reaching the photosensitive element.

[3] This ratio, P_m/P, is the "opacity." The natural logarithm of the opacity is the "extinction" (E) and is therefore related to the optical density as follows:

$$E = ln(P_m/P) = 2.303 \cdot \log(P_m/P) = 2.303 \cdot D$$

Conversely:

$$D = 0.4343 \cdot E$$

For theoretical purposes it is preferable to consider the extinction but for most experimental work it is far more convenient to express the results as optical densities.

all extensively for the study of suspensions of microörganisms is the "nephelometer" of Kleinmann (1920). This is a visual comparison instrument, bearing a superficial resemblance to the familiar plunger type colorimeter. It was used by von Angerer (1924a) in an attempt to determine the size of bacteriophage particles, and to him (1924b) we are indebted for the most extensive discussion to date of the optical properties of bacteria. This same instrument was used by Muntner (1926) to determine the bacterial concentrations in vaccines, by Liese (1926) to determine the surface area of suspended cells, and by Vignati and Schnabel (1928) for the detection of coagulation produced by disinfectants.

Valuable though the contributions made with the Kleinmann nephelometer have been, however, certain objections and limitations to its use should be pointed out. The instrument is designed to compare the intensity of the light scattered by the experimental suspension with that of the light scattered by a standard suspension of some sort. When standards are used there is, as already mentioned, a considerable possibility of introducing systematic errors arising either from the method used in their preparation or from subsequent changes, and these errors may easily pass undetected.

As in all instruments depending on the visual matching of two light intensities, subjective errors can be kept at a low level only at the cost of fatiguing repetition of measurements and of careful dark adaptation of the eyes. The maximum range of intensities which can be compared is 1:4, and for the most accurate results the two intensities should be much closer than that. This necessitates either a closely graded dilution series of standards, or the dilution of the sample to be measured, with the risk in either case of introducing pipette or sampling errors. The use of cups and plungers imposes a further general limitation. As it is not practicable to sterilize this assembly, the instrument can only be used for the measurement of killed cultures, or for living ones when freedom from contamination is of no importance. A photoelectric cell tyndallmeter was constructed by Frank for measuring yeast crops. It is briefly described by Gurwitsch (1932), but does not appear to have been widely used.

The number of densitometers which have been used for this purpose is much larger. The Pulfrich photometer, which is a visual comparison instrument, has been employed by Yen and Szu-Chih (1934) for following the effect of ultra-sonic waves on suspensions of bacteria, but, in general, objective instruments have been favored. All instruments of the objective densitometer type may be considered as being composed of three parts; a source of light, an optical system, which includes some transparent vessel to contain the suspension being measured, and a photometric unit.

The Moll (1920) "extinctiometer" (now listed as a "colorimeter and nephelometer for white light") was used by Noyons (1921) to follow the course of hemolysis, in a purely empirical manner, and by Strauss (1930) to estimate the number of bacteria per cubic centimeter. In this instrument the optical system produces two collimated beams of light. These are incident normally on two parallel walled glass cuvettes in which the suspension and the suspension medium alone are respectively contained. The more or less undeviated light transmitted by the two cuvettes is then directly focussed on the receivers of two thermopiles. These are connected in a bridge circuit in which a galvanometer is used to indicate the point of balance.

A simpler thermocouple instrument was constructed by Williams, McAlister and Roehm (1929) for the counting of suspended yeast cells. The light source was a battery-operated automobile head-light lamp. The optical system was similar to that of the foregoing instrument, but single, and a single junction thermocouple was used as the photosensitive element, the current output being read directly with a galvanometer and scale.

More recently two instruments have been designed in which the suspension to be measured is contained in a pyrex test-tube which also functions as a condensing lens of somewhat indeterminate characteristics. No other lenses are used, but there are diaphragms on each side of the tube. The first of these instruments was constructed by Richards and Jahn (1933) for counting yeasts and protozoa. The source of light is a 100 watt projection lamp operated directly from the 110 volt supply. A single Wes-

ton "Photronic" cell is used as the receiver and the output of current read directly on a small microammeter. In the more refined modification designed by Stier, Arnold and Stannard (1934), the lamp fluctuation is largely compensated by the use of two beams of light, two tubes, and two vacuum type photoelectric cells in a bridge circuit as in the Moll instrument. A galvanometer of moderate sensitivity is used as a null indicator. This instrument appears to have been mainly used for the preparation of yeast suspensions of accurately known turbidity for use as experimental material in studies of respiration and fermentation. For such purposes instruments of this general type have the evident advantage of simplicity and of avoiding contamination of the culture or the necessity of sampling. As will be seen later, however, the nature of the optical system would seem to limit their possibilities in other directions.

The instrument described in this paper is a photoelectric cell densitometer employing vacuum-tube amplification to increase the sensitivity. It was constructed early in 1933 as the outgrowth of a considerable experience with photoelectric cell photometers in the densitometry of photographic images, and in studying the kinetics of the extraordinarily rapid changes in scattering and in pigment absorption which take place in leaves, and still more strikingly in marine algae, when they are acted upon by heat or by chemical reagents. This experience indicated that a photometer could be constructed having a higher sensitivity and precision than previous instruments used to estimate the turbidity of suspensions of cells. It also led to the hope that with an instrument of this type the kinetics of such processes as growth, bacteriostasis, and bacteriophage lysis could be studied intensively in a manner not hitherto feasible. In the above mentioned study of marine algae, reactions involving extinction changes of relatively great magnitude, and often completed in less than one minute, were successfully followed by determinations made at intervals of ten or even five seconds.

The general features of the instrument, and the method of operation, conform closely to those already found satisfactory for the work just mentioned. The design of the optical system has a

considerable formal resemblance to those of Moll and of Williams, McAlister and Roehm. Actually, it was based on the type of optical system very generally used in the densitometry of photographic images when it is desired to measure the "specular" density, as distinguished from the "diffuse" density (cf., Tuttle, 1926). It was particularly influenced by an instrument constructed by Dreosti (1931) for observation of the transmission characteristics of "optically thin" suspensions of mastic particles. Dilute suspensions of large mastic particles have certain points of resemblance to suspensions of bacteria, and the reasons for Dreosti's design would seem to apply equally well in their case.

The somewhat simplified form of his optical system which was used in this first instrument has been productive of excellent sensitivity and linearity. Before proceeding to a detailed description of the instrument, however, it will perhaps be best to consider briefly the rather limited amount of theory bearing on the scattering of light by bacterial suspensions, and its application to their densitometry.

OPTICAL THEORY

Von Angerer (1924b) has pointed out that from general considerations the intensity of the light scattered laterally by suspensions of bacteria could be expected to be a function of their number per unit volume, their size, shape, the nature and state of their cytoplasmic contents (index of refraction relative to the medium, vacuoles, granules, spores, coagulation, pigment). As the result of an experimental study of suspensions of mastic particles of various sizes approximating those of bacteria, he found that the intensity of the light scattered at right angles to the incident beam appeared to be directly proportional to the number of particles per unit volume and to the square of the radius of the particle, rather than to the sixth power of the radius as would be required by Rayleigh's well known equation for scattering by particles small in relation to the wave-length of the light used for observation. Von Angerer suggested that spherical microörganisms could be expected to scatter light in this same way, and this important relation has been confirmed experimentally to a

fair degree of approximation by Liese and by Muntner. The significance of this expression of von Angerer's for optical density measurements will be further considered in a following paper by Mestre and Beard in connection with some observations on bacterial growth.

The previously mentioned experimental work with marine algae has led the writer to the belief that the scattering of light by living cells is in the main due to reflection and refraction at interfaces where there are differences in the indices of refraction, rather than to scattering of the Rayleigh type attributable to small particles in the cytoplasm. The appearance of suspensions of bacteria and other microörganisms under the microscope suggests that they should primarily be regarded as suspensions of micro-lenses, of shapes, sizes, and transparencies characteristic of the organism and its physiological state. Due to the relatively low ratio of the indices of refraction of the microörganisms and of their suspension medium (probably not over 1.390/1.335) these micro-lenses would have a low surface reflectance and be of a relatively extremely long focal length. The distribution of the flux incident on a single cell might consequently be expected to be characterized by an extraordinary anisotropy with almost all of the light scattered in a forward direction and only slightly deviated from its original direction.

It has already been remarked above that in any actual densitometer the transmitted flux entering the photometer unit will include a more or less large amount of scattered flux along with the undeviated flux. Denoting this fraction of the total scattered flux by q, and using the previous notation for the fluxes involved, we may write:

$$P = P_u + qP_s \tag{2}$$

It is evident that the magnitude of q will depend both on the directional selectivity of the optical system and on the angular distribution of the scattered flux. The directional selectivity can of course be calculated for any instrument with an optical system where the constants can be determined, but we know practically nothing about the angular distribution of the flux

scattered by suspensions of cells of various concentrations except that much more of it would appear to be scattered in a forward direction than in the case of ordinary turbid media. With the hope of providing a better foundation for the design of photometers for the study of such suspensions, and for the interpretation of the data secured by their use, a theoretical treatment of this problem has recently been undertaken.

By combining equations 1 and 2 we have:

$$D = \log P_m - \log (P_u + qP_s) \tag{3}$$

from which it can be seen that for any given suspension the value of the experimentally determined optical density will depend on the amount of scattered flux which is measured by the instrument along with P_u. The less the directional selectivity of the optical system the greater this amount will be, and hence the lower the value obtained for D for that suspension. It is also evident that this decrease in D will be proportionately much greater at higher concentrations, when P_u has a small value and P_s is large, than at low concentrations where the reverse is the case. A further point to be considered is that while the magnitude and angular distribution of P_s is to a considerable extent dependent on the cytoplasmic content of the cells through which it has passed, P_u is independent of these factors.

Certainly it would seem indicated that the practical procedure in densitometers is to exclude as much as possible of the disturbing flux P_s from measurement. This, Dreosti succeeded in doing to a highly satisfactory degree by careful collimation of the monochromatic incident beam and by a close directional selection of the flux transmitted to the photometric unit. In this way he was able, in the case of mastic suspensions, to keep q so small that not until the concentration of the mastic had been increased to a point where P was reduced to about $P_m \times 10^{-5}$, that is to say $D = 5$, did qP_s become large enough to cause P to differ significantly from P_u. Until this point was reached Dreosti found that the optical density was directly proportional to the concentration of the mastic.

Dreosti's results were obtained by measuring serial dilutions of a given suspension, so that the particle size remained constant. Under these conditions the variation in concentration actually only represents a variation in the number of mastic particles per unit volume. While it is customary to state that such results constitute evidence that Beer's law is obeyed, it should be emphasized that this is a very special sort of obedience to this law, limited to "particle concentration" and carrying with it no implications that if the concentration is varied in any other manner the law will still hold. If this limited form of Beer's law is obeyed by suspensions of microörganisms we could write:

$$D = sN \tag{4}$$

where N is the number of cells per cubic centimeter, and all factors, other than number, which may affect the absorption or the scattering power of the suspended cells are included in the proportionality constant s, which has the significance of a coefficient of scattering plus absorption per cell.

It is evident that s can have a constant value only as long as these other factors do not change, and as long as $P_u + qP_s$ does not differ significantly from P_u. With proper precautions the first condition can essentially be met in a dilution series, so that this method can be used to determine the range over which Beer's law is obeyed with any particular suspended organism and any given optical system. The results of such a test of the linearity of the present instrument will be given below.

In concluding this discussion of the theoretical aspect of the measurement of the optical density of suspensions of microorganisms, it should be emphasized that the values found for D for any given suspension will depend not only on the optical characteristics of the suspension but on those of the instrument. Further progress in the interpretation of optical density values will therefore depend not only upon the development of the theory of the transmission of light by such suspensions but also upon the extent of our knowledge as to the particular elements of the transmitted flux which are measured by any given instrument under any set of circumstances. In this latter necessity lies the main

reason for advocating the use of well defined optical systems whenever it is desired to apply theoretical considerations to the interpretation of experimental data.

DESCRIPTION OF THE INSTRUMENT

The optical portion of the instrument will be seen in plan in Figure 1. The source of light (S) is a 50 cp. 6 to 8 volt automobile headlight lamp (Mazda #1183), with its filament turned edge on to the collimating lens (L_1). It is operated at approximately 5.5 amperes as this has been found to give a satisfactory combination of luminosity and life. It has been found convenient and usually quite satisfactory to run the lamp from a single 6

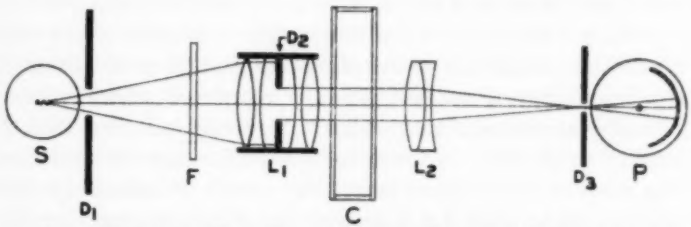

FIG. 1. SCHEMATIC PLAN VIEW OF OPTICAL SYSTEM OF DENSITOMETER

S, lamp; D_1, lamp diaphragm; F, heat resisting glass filter; L_1, collimating lens; D_2, lens iris diaphragm (the lens shutter has been omitted from the sketch); C, parallel walled glass cuvette for suspensions; L_2, lens to focus image of lamp filament on diaphragm; D_3, photocell diaphragm to exclude scattered light from photocell; and P, vacuum type photoelectric cell.

volt battery floating on a 5 ampere charging current, so that the battery is only supplying about 0.5 ampere to the lamp. If the power line is very heavily overloaded and the voltage is subject to large sudden variations, it will be necessary to operate the lamp from an equivalent bank of storage batteries discharging in parallel.

Between the lamp and the collimating lens are found the lamp diaphragm (D_1), which improves the quality of the collimated beam by screening out a considerable amount of scattered and reflected light, and a filter (F) of Corning "Heat-absorbing, medium shade" glass. This removes much of the infra-red and longer wave-length red emission of the lamp, thereby reducing

heating of the suspension and photocell, as well as rendering the beam somewhat more monochromatic. The importance of the degree of monochromaticity and of the reduction of the mean wave-length to a point where it is less than the diameter of the cells under observation will be discussed in the theoretical paper mentioned above.

The collimating lens is a "rapid rectilinear" type camera lens having a focal length of approximately 185 mm. and an aperture of 23 mm. It is provided with a shutter and iris diaphragm (D_2). This gives a simple method of controlling the magnitude of the flux P_o and of adjusting the diameter of the collimated beam to the size of the cuvette. Usually apertures of from 5 to 10 mm. have been used which, with a proper cuvette, would permit working with 2 to 3 cc. of suspension. Smaller beams could be used if the sensitivity of the photometric unit, or the intensity of the beam, were increased, thereby making it possible to use still smaller amounts of suspension. It should be noted that this method of controlling P_o must not be used for minor adjustments during a series of measurements, but that a standard aperture should always be used if it is desired to obtain comparable values for D. A method for making minor adjustments is given below.

The achromatic lens (L_2) has a focal length of 120 mm. and is adjusted to bring the collimated beam to a focus in the plane of the diaphragm (D_3), behind which the photoelectric cell is located. The aperture in this diaphragm is shaped to conform as closely as possible to the image of the lamp filament, and is approximately 1.2 x 3.7 mm. All light, therefore, deviated more than 1.2 mm. laterally, or 3.7 mm. vertically, through contact with suspended cells will be positively excluded from the photosensitive element. Expressed in terms of angular deviation this is approximately 0°35′ laterally and 1°45′ vertically so that the directional selectivity is fairly satisfactory, especially when it is considered that much of the light having lateral or vertical deviations between 0° and those just given will also be excluded.

In this preliminary instrument the constants of the optical system were determined by the fact that certain lenses happened to be available, rather than by design. While, as will be seen below,

the above optical system has resulted in a comparatively satisfactory degree of sensitivity and linearity, it should be pointed out that the directional selectivity can readily be increased thereby presumably increasing the sensitivity, the linear range, and, by further exclusion of P_s, reducing the response to factors influencing that flux.

The type of vessel used for containing the suspension has already been indicated. Various sizes and shapes can be used to meet different conditions. For experiments necessitating the measurement of small samples removed from a large culture, very small cuvettes can be used as previously pointed out. For the direct continuous observation of cultures where growth, stasis, agglutination, or lysis, are being studied larger cuvettes are more suitable as the introduction of stirrers and other accessories is thereby made possible. For very dilute suspensions the optical path, and hence the sensitivity, can readily be increased by the use of a cuvette of suitable proportions.

Richards and Jahn, as well as Stier, Arnold and Stannard, have objected to the use of special cuvettes for the measurement of suspensions on the grounds that cultures transferred to them for this purpose are decidedly liable to contamination. This objection would seem to be entirely justified if the purpose of the measurement is to determine, without sampling, when a growing culture has reached a certain desired turbidity, but for other purposes it is difficult to see why a parallel sided cuvette should not be used. The two most useful experimental techniques would appear to be the measurement of killed samples, or whole cultures, and the continuous intensive measurement of a single culture. In the first case contamination is not an issue. As for the second, it may be remarked that it is quite possible to sterilize a parallel sided cuvette, and keep a culture growing in it free from contamination during an experiment of any length.

As cuvettes of the above mentioned commercial quality are not optically perfect, it is necessary that when removed they should always be replaced in exactly the same position with respect to the beam. This is readily accomplished by the use of a close fitting guide. A region should be selected for the passage of the

beam where the opposite walls are sufficiently plane and parallel so that the image of the lamp filament is not displaced with respect to the aperture of the photocell diaphragm. No trouble has so far been found in doing this.

The main requirements for the photoelectric cell are that it have a linear response to changes in the amount of the incident flux over the range encountered, that it be free from fatigue, and that the base leakage be not too high. In addition as high a sensitivity as possible is desirable. These conditions are all met by the modern vacuum type cell, especially if the base leakage is controlled by sealing the cell in a moisture-tight housing in which a small amount of suitable drying agent is also enclosed. In the instrument here described, a 71-AV cell of the G-M Laboratories, Inc., was used.

In the case of instruments where the directional selectivity is not high, the flux reaching the photometric unit is relatively large and it is possible to measure the resulting photoelectric current directly with a not inconveniently sensitive galvanometer, as has been done by Stier, Arnold and Stannard. With a highly selective optical system, the amount of flux reaching the photocell is so reduced that amplification of the photoelectric current is necessary if the use of a high sensitivity galvanometer is to be avoided. This is clear when we consider that in the instrument of Stier, Arnold and Stannard, the photoelectric current from a 71-AV cell, presumably corresponding to the flux P_m, is reported as being 5 microamperes, while in the instrument here described, the corresponding current with the same type of cell is of the order of 0.05 microamperes.

The necessary degree of amplification may easily be secured with a single tube circuit of the type commonly employed in D.C. vacuum tube voltmeters, the voltage measured in this case being that produced by passing the photocurrent through a coupling resistance of high value in the grid circuit. The amount of amplification required will depend on the sensitivity of the microammeter or galvanometer employed in the output circuit. Both for speed and for accuracy it is necessary that the circuit should have a high degree of stability. This can be obtained in a simple

and satisfactory manner by the use of plenty of battery capacity and tubes of low current drain, or one of the many compensated circuits described in the literature may be employed. In general two alternative methods may be employed for the actual measurement; we may read the changes in the current output directly on a meter of appropriate sensitivity, or we may use a galvanometer as a null indicator, restoring the system to balance by a potentiometer in the grid circuit which gives us a direct measure of the voltage drop across the coupling resistance. This second method is less rapid but is to be preferred for measuring the density of cell suspensions, where observations do not need to be made at intervals of less than one minute, as with this system there is no dependence on the characteristics of the amplifier. In the second paper of this series there will be found a description of such a circuit and the method of operating it. In the instrument used for most of the experiments described in the next two papers the first method was employed, as an amplifier of this type, designed for the high reading speeds required by the previously mentioned work on leaves and marine algae, was available.

The circuit and its constants are shown in figure 2. The photocell, vacuum tube and grid circuit were fully shielded, but other shielding was not found necessary in the location where the instrument was used. The tube and coupling resistance were sealed in a moisture-tight container with a drying agent. The photocell was supplied with a potential of 90 volts by the dry battery B_1. As this is well above the saturation voltage for this cell, the maximum reduction in the applied potential of about one volt caused by the I x R drop in the 20 megohm coupling resistance (R_1) is without disturbing effect on the linearity of the light flux/photo-current characteristic of the cell. The type '33 screen grid battery pentode was supplied with filament current by the three cells of an automobile storage battery (B_3) discharging in parallel, and the grid was biased at -3 volts by two dry cells (B_2). A potential of 45 volts was applied to the screen grid and of $22\frac{1}{2}$ volts to the plate by a heavy duty "B" battery (B_4), these values having been found to give a satisfactory combination

of sensitivity and moderate current drain. Lower potentials failed to give the desired increase of one milliampere in the plate current for a decrease of approximately one volt in the grid bias. With the photocell dark, the steady plate current of approximately 4.3 milliamperes passing through the multi-range Sensitive Research Instrument Corp. milliammeter (M) is balanced out by adjusting the resistances R_2 and R_3.

With such a meter, having the three full scale ranges of 10, 1, 0.1 milliamperes, the operation of the instrument is much facili-

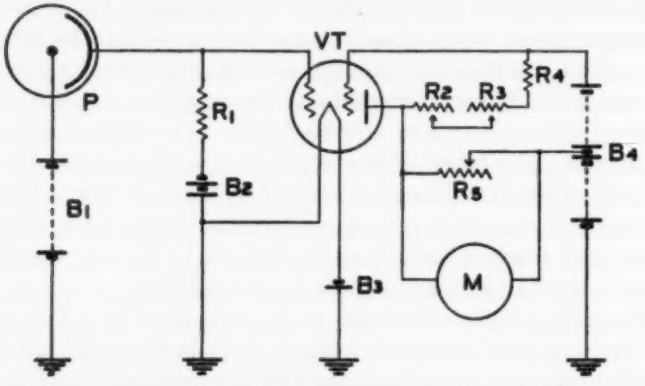

FIG. 2. SCHEMATIC WIRING DIAGRAM FOR PHOTOMETRIC CIRCUIT

P, photoelectric cell; B_1, 90 v. dry "B" battery; R_1, 20 megohm coupling resistance; B_2, 3 v. (two dry cells); VT, '33 power amplifier pentode; B_3, 2 v. battery (3 cells of automobile storage battery connected to discharge in parallel); R_2, 100 ohm variable resistance; R_3, 2500 ohm variable resistance; R_4, 4000 ohm fixed resistance; R_5, 1000 ohm variable resistance; B_4, 45 v. heavy duty "B" battery, center tapped; and M, multi-range microammeter.

tated. With the meter on the 10 ma. range, and the shutter of the collimating lens closed, the steady plate current is approximately balanced out in the manner just noted. Shifting successively to the 1000 and 100 microampere ranges, the zero can be adjusted to within 0.1μa. With the meter again on the 10 ma. range the shutter of the collimating lens is opened and the cuvette removed from the beam, allowing the flux P_o to fall on the photocell. With the iris diaphragm of the collimating lens the meter reading is brought close to 1 ma. and the adjustment completed

on the 1000μa. range with the aid of the shunting resistance R_s.[4] It is advisable to turn on the lamp and the amplifier twenty to thirty minutes before attempting to complete these settings to allow the system to come to thermal equilibrium. When the zero and P_o readings are stable, the meter reading corresponding to P_m is determined by sliding into the beam the carefully cleaned cuvette, filled with the suspension medium which is to be used. This determination is made with the greatest possible precision, with repeated checking of the zero and P_o readings, as it is not ordinarily repeated during a given set of observations.

With the cuvette filled with the suspension to be measured the meter reading corresponding to the flux P is now determined in the same manner as described for P_m, zero and P_o being read and, if necessary, reset before each P_m reading, and reread after it. The zero and the P_o reading will usually remain stable over considerable periods of time with slow random fluctuations of the latter of the order of a few microamperes. Under some conditions of the lamp batteries or of the power line there will be a slow even drift of the P_o reading. This may be compensated for by making the setting for P_o so that the readings before and after that of P will differ from 1000 μa. by an equal amount. With the above mentioned meter, with its 5-inch 100-division scale, knife-edge pointer, and mirror to reduce parallax errors, it was found possible to make readings with a precision of one to two μa. Accurate reading of the scale is aided by illuminating the scale and mirror through a sheet of ground glass large enough to provide a uniform background at any position of the pointer. The precision naturally increases as the value of P, and hence the effect of the variations in P_o, decreases.

The values of the readings obtained in the above manner for P and P_m are now corrected for the overall departure from lin-

[4] It should be noted that the adjustment of the instrument to give a reading of 1000 μa. for P_o is entirely arbitrary and is done for convenience in setting and to utilize as much of the meter scale as possible. In the present case, also, the calibration curve (see below) from which the meter readings are corrected is based on this particular range. Any other standard value for P_o could have been selected, and the instrument calibrated accordingly, as the optical density, being the logarithm of the ratio P_m/P is independent of the absolute magnitude of P_o.

earity of the amplifier and meter over the fixed output range determined by the settings for zero and P_o, it being assumed that under the operating conditions given the photoelectric current is directly proportional to the incident flux over this range. These corrections are made from a calibration curve obtained by impressing on the grid a series of voltages which are arbitrary fractions of the voltage required to produce an increase of 1000μa. from the steady plate current with the photocell dark. With the above circuit the maximum departure from linearity is of the order of 7 μa. at the middle of the 1000μa. range, decreasing symmetrically as zero and full scale deflections are approached.

Assuming that these corrected readings are proportional to the corresponding fluxes, it is apparent that to obtain D we need only subtract the logarithm of the reading for P from that for P_∞, as the proportionality constant cancels out. The handling of the experimental data is shown in the first 5 columns of table 1. When using this procedure densitometric determinations are highly objective, the subconscious evidently being entirely unable to lend a hand at deciding which of two meter readings might give the more desirable value for D.

LINEARITY, PRECISION, AND ACCURACY

It has already been mentioned that the determination of the optical density of the members of a series of dilutions of a given suspension may be used as a method of discovering the extent to which the "particle concentration" form of Beer's law is obeyed for any particular instrument and type of suspension. In any range over which this law holds, s, in the expression $D = sN$, will be constant, and if the various values for D are plotted against the corresponding concentration values a straight line will result. Over this range the instrument may be said to be linear for D and N for the type of suspension examined.

The data shown in table 1 represent the results of measuring a dilution series prepared from a seventeen-hour peptone culture of *Escherichia coli*. In carrying out such a series the utmost precautions in sampling and care in pipetting are necessary. It is, moreover, essential that there be no growth, or other change, in

the suspended organisms during the test. This necessitates the addition of a suitable bacteriostatic agent to the primary culture. In the series here recorded growth was stopped by the addition

TABLE 1

Serial dilution of a suspension of Esch. coli

FLUX	μa. (OBS.)	μa. (CORR.)	LOG (μa-CORR.)	D (OBS.)	D (CALC.) $= D_o \times N/N_o$	ΔD	ERROR (PER CENT D)	ΔD FOR. $\pm$ 1μa.	ERROR-PER CENT D FOR $\pm$ 1 μa.
P_o	1000	1000							
P_m	861	863	2.9360						
P for $N/N_o = 1$	280	283	2.4518	0.4842	0.4842	0.0000	0.0	0.0015	0.3
1/2	486	493	2.6928	0.2432	0.2421	+0.0011	+0.5	0.0009	0.4
1/3	587	594	2.7738	0.1622	0.1614	+0.0008	+0.5	0.0007	0.4
1/4	647	653	2.8149	0.1211	0.1211	0.0000	0.0	0.0007	0.6
1/8	747	752	2.8762	0.0598	0.0606	−0.0008	−1.3	0.0006	1.0
1/16	803	806	2.9063	0.0297	0.0303	−0.0006	−2.0	0.0005	1.7
1/32	830	833	2.9206	0.0154	0.0151	+0.0003	+2.0	0.0005	3.3
1/64	847	849	2.9289	0.0071	0.0076	−0.0005	−6.6	0.0005	6.6

In the first column P_o represents the flux reaching the photocell with no cuvette in the beam, P_m the flux transmitted by the cuvette filled with the same medium in which the organisms are suspended, and P the fluxes transmitted when the cuvette contains the various dilutions represented by the ratio N/N_o. N_o, the number of cells per cubic centimeter in the undiluted suspension, was not directly determined in this case but was approximately 6×10^8 per cubic centimeter, so that the dilution series extended to a little below 10^7 per cubic centimeter. The second column gives the observed reading in microamperes corresponding to the various fluxes, and the third column these readings corrected for the over-all non-linearity of the amplifier and meter as explained in the text. The fourth column contains the logarithms of these corrected readings, and the fifth the optical densities (D) obtained by subtraction of the logarithms of the readings corresponding to the various values of P from the logarithm of the reading for P_m. The sixth column gives the D values obtained by multiplying D_o, the optical density of the undiluted suspension, by the various values for N/N_o, and the seventh column the differences (ΔD) between these values and those determined experimentally. Assuming that the values calculated from the dilutions are correct, the eighth column expresses these values for ΔD as per cent error in the determination of D. In the next to the last column are shown the ΔD values which would result from an error of ± 1 μa. in reading the meter, and in the last column the percentage error in the determination which would result from this observational error. The recorded errors evidently do not materially exceed the errors which might result from this degree of observational precision, which is maximal for the arrangement of the instrument used in this series.

of KCN and the suspension allowed to become optically stable before the dilutions were made.[5] These also were checked for stability. The dilutions were made with the same lot of medium used for the primary suspension.

By comparing the columns for D computed from the observations and for D calculated by multiplying the optical density of the primary suspension by the various concentration ratios, it can be seen that the instrument is essentially linear over the concentration range of 64, or a density range of 0.477. The deviations of the observed D values from the calculated ones are apparently random and, as can be seen by reference to the next to the last column, are never significantly greater than those which would be caused by an error of $\pm 1 \mu$a. in the reading of the meter for the particular flux. This is the maximum precision of reading attainable with this arrangement of the instrument, and, under ordinary conditions it would, as already mentioned, be less than this at the lower concentrations where P is more affected by fluctuations in P_o which is the least stable factor.

It is quite evident from the last column of table 1 that the percentage error in the determination of D is increasing at such a rapid rate with decreasing concentration that, at concentrations much below 10^7 per cubic centimeter (for *Esch. coli*), the percentage error in the determination of D will become undesirably high unless the precision of reading can be increased. There would seem to be grounds for expecting that satisfactory precision at concentrations of 5×10^6 per cubic centimeter can be attained with moderate improvements in the instrument and that, eventually, it may be possible to work down to 10^6 per cubic centimeter. While such a downward extension of the effective range would be very desirable for some purposes, it should be pointed out that the present minimum is sufficient for most physiological problems. In studies of bacterial metabolism, for instance, it has usually been found necessary to start the experiment with concentrations of from 5×10^6 (Martin, 1932), to 1.0 or 1.5×10^7 per cubic centi-

[5] The effect of various concentrations of KCN on the optical density of suspensions of *Esch. coli* will be discussed in the following paper by Mestre and Beard.

meter (Walker and Winslow, 1932), in order to obtain measurable changes in the substrate material or metabolite being investigated. Most of the phenomena of the growth cycle, and of lysis, bacteriostasis and disinfection can be studied directly at concentrations above this level, and in many of the remainder valuable data can be obtained by accurately timed measurements when growth has brought the concentration to optically measurable densities.

The linearity of the range of this instrument above $D = 0.500$ was not determined with the same care as the range below that density as it was not important for the immediate experimental purposes. Such dilution series as were made from older cultures which had reached higher optical densities, indicated a slowly progressing departure from linearity in a negative direction (decreasing s), becoming more rapid around $D = 0.650$ or 0.700. This was expected on the basis of the theoretical considerations already stated and conforms to the experience of other workers (cf., Strauss; Stier, Arnold and Stannard; loc. cit.). There is reason to believe that by further increase in the angular selectivity of the optical system and in the monochromaticity of the light used the linear range may be extended upward to include the whole range usually encountered in natural bacterial growth. It should be pointed out that it is quite possible to work in this region of higher concentrations by the use of a calibration curve, or Strauss' procedure may be followed, a single dilution being made to bring the suspension within the linear range.

In the next to the last column of table 1 we have seen that a precision of $\pm 1\mu a.$ in reading the amplified output of the photoelectric cell results in a precision of determination of D varying from $\pm$ 0.0015 at a D of 0.4842 to ± 0.0005 at a D of 0.0076. This might also have been expressed as a precision of determination of the opacity (P_m/P) of approximately 0.4 and 0.1 per cent at the same two values of D. As seen from the last column both of these ways of expressing the precision tend to give an erroneous impression of what may be termed the useful precision of the instrument, that is to say of the percentage error which we may expect the instrument to contribute to the determination of D, which is what we are really interested in knowing.

In this connection it is important to bear in mind the distinction between precision and accuracy. To quote from Laws (1917) "we must remember that a high precision, or agreement of the results among themselves, is no indication that the quantity under measurement has been accurately determined." In the determination of the D of a suspension of cells there are many sources of error other than lack of instrumental precision. Errors of considerable magnitude may arise from faulty manipulation or through unnoted changes in the physical constants of the instrument. An accuracy in the determination of D closely approximating the limit set by the precision of the instrument is only to be achieved through care and skill in operation, and the higher the precision the more important these factors become.

In addition to errors in the determination of the optical density of the suspension placed in the instrument for measurement, it should be pointed out that, depending on the experimental technique employed, various other errors may enter to affect the accuracy of the determination. Three of the techniques which have been found most useful will be described in the following paper by Mestre and Beard and the advantages and difficulties peculiar to them discussed.

SUMMARY

The literature reveals a rapid increase in the use of objective photometers for measuring the turbidity of suspensions of microorganisms. A distinction is drawn between "tyndallmeters" with which the turbidity is estimated by observation of the intensity of the scattered flux, and "densitometers," in which the transmitted flux is measured. Various instruments of both types which have been used for this purpose are briefly described, and the limited amount of available theory relating to the scattering of light by suspensions of microörganisms is discussed with especial reference to instrument design and the interpretation of experimental data. Based on this theory and on previous experience in the densitometry of spectrophotographic negatives and in the photometric study of the kinetics of rapid changes in scattering and pigment absorption in plant material, a photo-

electric cell densitometer of relatively high precision has been constructed for the study of the kinetics of growth, stasis and lysis in suspensions of microörganisms. The design and operation of this instrument is described and its characteristics briefly discussed.

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THE MICROBIAL CONTENT OF SOFT WHEAT FLOUR[1]

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Numerous investigations have shown the microbial flora of flour to be extremely heterogenous, including molds and yeasts as well as bacteria. The amount of microbial development depends upon the moisture content of the flour. Thom and LeFevre (1921) showed that a moisture content of less than 13 per cent inhibits growth of all organisms. According to Arnoldow (1908) most bacteria and molds grow in flour when the moisture content is greater than 17 per cent. The bacterial content may reach such enormous numbers as 310,000 to 5,200,000 per gram according to Turley (1922). Kent-Jones and Amos (1930) demonstrated that freshly milled patent flour may contain 20,000 or even less organisms per gram, while lower grades may contain 300,000 or less. These investigators found also that storage of flour decreases the microbial content; the rate of decrease depending upon the moisture content and storage temperature.

Sometime ago a southern milling company reported difficulty regarding the keeping quality of its flour. Since rancidity developed rapidly, a bacteriological investigation was made, to determine why this particular product lacked the keeping qualities of other flours on the market.

METHODS OF PROCEDURE

Ten-gram samples of wheat and flour, collected from the important milling stages, were thoroughly shaken in liter bottles of sterile water for at least one-half hour to insure uniform suspensions before making further dilutions and plating. Nutrient

[1] Report of an investigation conducted in the laboratories of the Department of Bacteriology, University of Tennessee, Knoxville.

agar was used, and incubation was conducted at 37°C. for forty-eight hours for determination of bacterial content, and for one week at 37°C. for determination of mold content.

Samples of wheat and flour from the mill were collected and analyzed on three separate occasions. A bacteriological analysis of a sample of flour which had been in storage for several months, was also made. Later, the microbial content of 21 other flours, obtained on the open market, was determined. These 21 brands were the products of mills in the South and Middle-west.

RESULTS

The microbial content per gram of flour at various stages in the milling process is shown in table 1.

TABLE 1

Microbial content per gram of flour at various stages in the milling process

STAGE OF PROCESS	BACTERIA*	MOLDS*
Cockle cylinder	3,200,000	7,000
Scourer	500,000	5,500
Wheat washer	210,000	3,000
Wheat dryer	60,000	2,600
First break rolls	52,000	2,400
Second break rolls	56,000	2,400
Third break rolls	50,000	2,200
Purifier	44,000	1,800
First smooth rolls	35,000	2,000
Second smooth rolls	32,000	1,800
Chlorinator	21,000	1,700
Flour bin (fresh)	23,000	1,800
Warehouse (storage)	337,000	30,000

* Average counts on samples collected on three separate occasions at weekly intervals.

The microbial content of twenty-one brands of flour obtained on the market proved to be quite uniform. The bacterial content ranged from 3100 to 7500 per gram, and the mold content varied between 100 and 640 per gram.

DISCUSSION OF RESULTS

There was a definite decrease in the microbial content of flour in its progress through the various stages of milling. The average

of 23,000 bacteria and 1,800 molds per gram should not be considered too high for freshly milled flour, but the large increase in numbers of microörganisms in the stored flour makes it evident that ideal conditions for development prevail during storage. Although a favorable temperature is necessary, it is probable in this case that microbial development during storage of the flour was due chiefly to presence of moisture derived from a humidifier used in the plant, since 21 other brands purchased on the open market showed a uniformly low bacterial and mold content. These flours may have been in storage for some time also, but if such is the case it is apparent that optimum conditions for microbial development were lacking.

The freshly milled flour was much lighter in color than other flours examined. This was probably due to over-bleaching, which may have allowed microbial or atmospheric oxidation to take place more easily, thus giving rise to rancidity.

No attempt was made to determine the identity of all of the organisms present, but some of the more prevalent types were those of the *Aerobacter aerogenes* and *Bacillus mesentericus* groups. Other genera frequently present were *Sarcina*, *Achromobacter*, and *Flavobacterium*. Occasionally agar-digesting forms related to *Cellulomonas* were found.

CONCLUSIONS

The microbial content of flour may increase considerably during storage if conditions for development are favorable.

If flour is over-bleached, oxidation of the fats may occur more readily.

Flours obtained on the market usually do not possess a high microbial content.

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THE MICROBIOLOGY OF THE UPPER AIR. II[1]

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The more common use of aircraft during recent years has enabled various investigators to make collections of microorganisms occurring in the air at various altitudes in widely separated parts of the world. Such studies have been reported in the United States by Stakman, Henry, Curran and Christopher (1923), Brown (1930), and Meier, Stevenson and Charles (1933). In Canada, Craigie and Popp (1928) have collected rust spores from the air and in Russia some microbiological studies in the upper air were made by Mischustin (1926). More recently Meier and Lindbergh (1935) have published the results of collections made by Colonel Lindbergh in extended flights above the Arctic Circle.

During the past two years collections of this nature have been made in the vicinity of Boston. This has been possible due to the fact that almost daily airplane flights to relatively high altitudes were being conducted under the direction of the Division of Meteorology, Department of Aeronautical Engineering, of the Massachusetts Institute of Technology. These flights were designed primarily for the purpose of obtaining meteorological data at high altitudes, but through the courtesy of the Division of Meteorology, the same flights have sometimes been used for making collections of air micro-organisms.

A description of the apparatus and the results of collections made in 45 earlier flights of this nature have been reported previously (Proctor, 1934). The present report deals with the

[1] Contribution No. 56 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

collections made on 30 additional flights carried out between April and November, 1934.

In brief, the collection apparatus was a brass plate capable of being rotated, with six holes drilled through it at equal intervals. In these holes were set disks of lens paper on a wire screen support. The disks were saturated with a hydrocarbon oil with a low congealing point (−71°C.) and acted as the actual trapping or filtering agents. The plate was enclosed in a tight metal drum with an inlet and outlet tube set on the opposite faces so that a current of air drawn through the outlet could pass through each of the six holes, one at a time, by rotating the plate.

The entire apparatus was sterilized by heat before use. During transportation between the laboratory in Cambridge and the flying field of East Boston it was placed in a special sterilized container.

When installed in the plane, the inlet and outlet tubes of the collector were connected to other tubes installed permanently in the plane so that a current of air could be drawn from outside the cabin through the collector and out through a Venturi tube on the roof, which furnished the vacuum necessary for moving the air. The collections could be made by merely turning a knob, thus enabling the sole occupant of the plane to control the collections, in addition to his numerous other duties as pilot and meteorological observer. Additional holes drilled in the rotating plate between the collection disks allowed the unrestricted passage of air through the apparatus when collections were not being made, thus furnishing what might be termed an air-wash of the apparatus between collections or exposures at different altitudes. One of the six collection disks was ordinarily not exposed and therefore acted as a control.

This apparatus does not collect all the micro-organisms from the air passing through it, but offers a means of comparison for collections at various altitudes. A perfect trap would so restrict the air flow as to make its use unsuitable in altitude flights of the nature of those mentioned. Tests made in the M.I.T. wind-tunnel indicated an air-flow through the device of approximately 1 cubic foot per minute under general flight conditions.

Some of the flights were made with a Curtis-Robin plane. The later ones were made with a Fairchild plane which has been assigned for meteorological work at the M.I.T. by the Aviation Corps of the U. S. Army. The flights were made between 7 and 9 a.m.

The laboratory work was usually carried out within a few hours after completion of flight. The oiled paper filters were removed to a specially sterilized glass cell, using aseptic technique. A sterile cover slip was placed over the disks and they were examined directly with a microscope, using a 16 mm. objective and a 10× ocular. By means of a mechanical stage, counting of the visible dust particles and any pollen grains present on the disks was simplified.

After the direct examination, the disks from each altitude were placed in separate tubes containing 10 cc. sterile water and plugged with sterile rubber stoppers, using aseptic precautions. After standing five minutes the tubes were shaken thoroughly until the paper disks were disintegrated into separate fibers. The water from each tube was divided among three or four Petri plates, being transferred by means of sterile pipettes. Standard nutrient agar was added in the usual manner, allowed to cool, and the plates incubated for five days at room temperature. At the end of this period they were placed in a 37°C. incubator for two additional days, after which counts were made.

This technique made possible determination of those organisms capable of growing aerobically on standard nutrient agar at room temperature, also those growing at 37°C.

Tables 1 to 6 contain the results obtained from 30 flights. In addition to the bacteria and mold plate counts, the results of the direct dust counts are included in the last 27 flights. Due to variation in conditions of flight, it was not possible to have all exposures of an equal time interval nor could the altitude brackets be identical.

Such other atmospheric factors as seemed pertinent have been included in the tables. It will be noted that in the temperature column the difference between G (ground temperature) and T (temperature at the top of flight) is often considerable. The

TABLE 1

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
1. April 30, Mon.	N Pc	SSW to W	0	55	G 6.8	10	5–10	4	1	
					T −37.1	11	11–15.5	9	0	
						10	16–20	5	0	
						22	20–25.4–16	15	1	
		Sm				5	15–3	4	1	
Control						0		0	0	
2. May 2, Wed.	N Pc from ocean	S	0	55	G 12.5	10	5–10.5	18	2	
		Cl, Sm			T −25.5	10	11–15.8	30	0	
						10	16–20	12	1	
						10	20.2–22.3	6	0	
						8	19–10	2	1	
Control						0		0	0	
3. May 8, Tues.	Pc	NW	0	57	G 10	10	5–10	1	2	
					T −22.8	10	11–15	1	2	
		SH				10	15.5–21	1	0	
		Waltham				8	0.6–1	7	57	
		Waltham				8	1–1	3	00	
Control						0		0	0	
4. May 11, Fri.	N Ta	W	0.04	87	G 25.3	8	5–10	32	10	641
					T −17.9	9	11–25.5	4	4	225
		Dust storm, strong upward currents				4	16–18	6	2	279
						10	17–2.8	68	3	927
						10	2.7–1.5	74	8	562
Control						0		0	0	110
5. May 12, Sat.	Pc	NNW	0	53	G 9.9	10	5–10	0	0	271
					T −33.6	10	10–15	1	0	235
		C				12	18–23–16	1	1	186
Control						0		0	0	58

Symbols used in tables 1 to 6 concerning weather refer to observations made at the time of the flight and are as follows: C, clear; Cl, cloudy, O, over-cast; F, fog; R, rainy; Sm, smoky; SH, slight haze; PCl, partly cloudy.

Some of these terms are difficult of strict definition but give an indication of general weather conditions.

The air masses are the results of the analysis of air mass properties determined from the meteorological data obtained on each flight in accordance with the classification of Willett (1933). The following symbols and their significance are explained briefly below.

Pe (Polar Canadian), from the northern continental area. Pp (Polar Pacific), from the north Pacific area. Pa (Polar Atlantic), from the Sargasso Sea area. Ta (Tropical Atlantic), from the Sargasso Sea area. Tg (Tropical Gulf), from the Gulf of Mexico and Caribbean Sea area. N, modified air masses originally of P or T character but of transitioned type.

The bacteria, molds and dust counts as given are the total numbers enumerated from each exposure.

rainfall cited is that precipitation which occurred during the twelve hours preceding each flight. The relative humidity is that determined at ground level at the approximate time of each

TABLE 2*

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
6. May 13, Sun.	N Pc becoming Pa	NNW	0	42	G 11.4	10	5.5–10.5	2	2	338
					T −27.8	7	13–16	1	0	270
						9	16.8–20	5	0	175
		C				20	20.5–23–20.5	2	0	105
Control						0		0	0	35
7. May 14, Mon.	Pp	W	0.02	78	G 14.2	9	5–10	1	0	487
					T −21.9	10	11–15.5	3	1	430
						12	16–20	3	3	310
		O				7	20.3–21.5–20	5	0	195
						8	18.4–10	1	1	225
Control						0		0	0	20
8. May 18, Fri.	N Pc	W	0.01	55	G 13.5	9	5–10	35	5	921
					T −24.7	10	11–15.5	3	0	572
						15	16–20	3	0	383
		P Cl				13	20–22–20	4	0	291
						8	19–10	3	1	178
Control						0		0	0	85
9. May 19, Sat.	Pc	N	0.02	44	G 13.7	8	5–10	7	0	338
					T −24.7	9	10.5–15.5	3	0	287
		C				9	16.0–21.0	0	1	215
Control						0		0	0	75
10. May 21, Mon.	N Pa	N	0	88	G 15.9	8	5–10	0	1	521
					T −17.3	12	11–15.5	0	0	1225
		O				15	16–20	1	0	475
						9	19–20–10	0	1	438
Control						0		0	0	40

* See footnote to table 1 for explanation of symbols

flight. The wind is also that occurring at the lower levels on the morning of the flight but may not be the same at all levels.

The data included in these tables suggest some of the numerous

factors which may play a part in the distribution of micro-organisms in the upper air. It is generally believed that heavy pre-

TABLE 3*

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
11. May 23, Wed.	N Pc	W	0.12	72	G 15.4	4	7.6–10	0	8	105
					T −20.3	12	11.5–15.5	1	8	167
		P Cl				11	16–20	5	4	71
						10	19–10	1	5	207
						4	9–3	4	4	255
Control						0		0	0	35
12. May 24, Thur.	N Pc	NW	0	49	G 13.8	9	6–10	5	0	145
					T −29.1	10	11–15	10	3	128
		P Cl				12	16–20	0	3	95
						18	20–23–20	2	1	72
						9	19–10	1	0	162
Control						0		0	0	60
13. May 25, Fri.	N Pc	NE to SW	0.01	72	G 11.5	8	5.5–10	2	1	450
					T −25.9	11	11–16	1	0	327
						13	16.2–20	8	1	185
	P					5	20–21–20	1	0	83
	Ta	O				5	19–10	0	1	430
Control						0		0	0	42
14. May 28, Mon.	Pc	Variable winds	0	60	G 16.4	10	5–10	1	0	84
					T −19.0	8	11–15	1	1	73
						8	16–20–18	0	0	65
		P Cl				8	14–3.5	0	2	87
Control						0		0	0	45
15. May 29, Tues.	N Pa	S	0	72	G 12.8	8	6.7–10	1	2	373
					T −19.6	13	11–15.5	0	0	435
	N Tp					12	16–20	4	0	237
		O				10	20.2–22–20	2	2	145
						6	19–9	2	4	210
Control						0		0	0	29

* See footnote to table 1 for explanation of symbols.

cipitation usually reduces the numbers of micro-organisms in the air. The lethal effects of low temperatures and sunlight on cer-

tain types of bacteria have long been recognized. The strength of air currents and their direction, as well as the origin of air

TABLE 4*

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
16. May 31, Thurs.	N Pp	W	0	74	G 21.5	10	4.5-10	7	1	137
					T −17.3	8	11-15	1	1	98
						12	15.5-21-20	2	2	75
		SH				10	19-10	4	1	82
						10	10-3	6	0	115
Control						0		0	0	27
17. Oct. 9, Tues.	Pc and Pp	NW	0	63	G 11.1	5	0.8-3.0	1	5	61
					T − 9.0	6	3.2-6.2	0	3	142
						6	6.5-9.5	2	1	80
		C				4	10-12	0	0	59
						5	12.2-15	10	0	96
						1	15-16	1	5	28
18. Oct. 10, Wed.	N Pc	NW	0	63	G 6.1	8	1-5	2	0	21
					T − 8.8	9	5.5-10	0	0	25
		C				9	10.5-15	2	0	67
						5	14-4	2	0	60
Control						0		0	0	20
19. Oct. 11, Thurs.	N Pc	W ?	0.01	71	G 10	5	0-2.8	1	3	155
					T −10.7	6	3-6	6	3	164
		R				6	6.2-9.2	1	2	502
						5	9.5-12	2	2	342
						6	12.2-15.2	3	3	128
Control						0		0	0	42
20. Oct. 15, Mon.	N Pc	W	0.08	97	G 6.4	3	2-3.5	3	0	150
					T −11.8	4	4-6	2	0	77
		R				4	7-9	1	1	61
						5	9.5-12	2	3	284
						5	12.5-15	2	2	232
Control						0		0	0	15

* See footnote to table 1 for explanation of symbols.

masses are likely to be of significance from the standpoint of the bacterial and fungal populations at the higher altitudes. The

humidity of the air may also influence the numbers of bacteria in the atmosphere above the earth. It is highly unlikely that any

TABLE 5*

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
21. Oct. 16, Tues.	Pc	N	0	86	G 6.6	6	2–5	2	0	20
					T −8.5	6	5.2–8.2	0	3	41
						6	8.5–11.5	0	1	18
						4	12–14	1	3	40
		O				4	14.2–16.2	1	0	27
Control						0		0	0	14
22. Oct. 17, Wed.	N Pc	W	0	85	G 8.8	4	1–3	1	4	175
					T −7.0	5	3.5–6	1	2	161
						5	6.5–9	1	0	180
		C				5	9.5–12	3	2	210
						5	13.5–15	0	2	141
Control						0		0	0	62
23. Oct. 19, Fri.	Pc	N	0	85	G 8.6	4	1.7–4.3	12	4	115
					T −12.4	1	5–6	1	0	60
						5	6.4–9	1	0	70
		O				5	9.5–12	1	2	210
						5	12.4–15	6	4	885
Control						0		0	0	45
24. Oct. 25, Thur.	Pa	NNE	0	77	G 7.6	6	2–5	2	1	148
					T −7.3	6	5.2–8.2	0	0	171
						6	8.5–11.5	3	1	170
		C				4	12–14	2	0	181
						4	14.2–16.2	3	0	242
						1	16.5–16.8	1	0	105
25. Oct. 26, Fri.	Pa	SW	0	97	G 8.2	4	1–3	2	1	79
					T −7.3	4	7–9	1	0	37
		Cl				6	10–13	2	1	75
						5	13.5–15	1	0	59
						2	5–3	0	0	47
Control						0		0	0	15

* See footnote to table 1 for explanation of symbols.

combination of these numerous influences is exactly identical on more than one occasion or at any particular altitude.

Reference to the tables indicates that on all of these 30 flights some bacteria were collected which were capable of growing on

TABLE 6*

Flight data

NUMBER	AIR MASSES	WIND, WEATHER	RAINFALL	RELATIVE HUMIDITY	TEMPERATURE	TIME	ALTITUDE (1000 FT.)	BACTERIA	MOLDS	DUST COUNTS
				per cent	*°C.*	*minutes*				
26. Oct. 30, Tues.	Pc	W	0			6	2-5	1	1	125
						6	5.2-8.2	0	0	258
						5	8.5-11	0	1	81
		C				4	11.2-14.2	0	0	108
						5	14.5-17	1	0	101
Control						0		0	0	40
27. Oct. 31, Wed.		W		69	G 4.7	4	1-3	2	5	152
					T −23.6	5	3.5-6	1	3	140
		P Cl				5	6.5-9	5	1	170
						5	9.4-12.2	1	0	125
						6	12.6-15.8	2	1	432
Control						0		0	0	28
28. Nov. 1, Thur.	N Pc	S	0.08	92	G 11.8	6	2-5	1	1	35
					T −14.4	6	5.2-8.2	2	1	41
	Tg					5	8.5-11	2	0	307
		R				4	11.2-14.2	5	0	833
						5	14.5-17	2	0	310
Control						0		0	0	61
29. Nov. 3, Sat.	Pc	W	0	71	G 1.9	5	2.5-5	0	1	97
					T −17.6	6	5-8	0	0	85
		Cl				6	8-11	1	0	80
						6	11.2-14.2	0	1	232
						7	14.5-18	1	0	218
Control						0		0	0	37
30. Nov. 5, Mon.	N Pp	SW	0.02	87	G 14.5	4	1-3	2	2	56
					T −5.2	7	3.6-6.1	1	0	61
		P Cl				5	6.6-9	1	0	32
						5	9.5-12	0	0	55
						4	13-15	0	0	35
Control						0		0	0	23

* See footnote to table 1 for explanation of symbols.

nutrient agar. In most of the flights bacteria were more numerous than molds, although it must be stressed that the culture medium used was not suitable for the growth of all molds.

Molds were obtained in all the flights except one, no. 18. Seven collections made at altitudes of 20,000 feet or above indicate that bacteria may be found at such altitudes, although the number obtained per minute of collection time (0.26) is lower than that value computed on the basis of all flight collections (0.38). Altitudes are far from being the only factor concerned in the distribution of micro-organisms in the upper air, as is shown in numerous instances when collections at the higher levels during the same flight have resulted in larger numbers of micro-organisms even after the time factors have been adjusted. Flight no. 7 offers an illustration of this nature.

The data obtained in flight no. 4 are of particular interest in view of the fact that on that day an unusual dust storm passed over Boston which was said to have originated in the mid-western drought areas. The bacterial counts were of greater magnitude than those usually observed and would seem to indicate that the increased incidence of bacteria occurred particularly at the lower levels. The mold counts obtained were also higher than in all other flights, with two exceptions. One of these exceptions was flight no. 3 which was conducted at an altitude of 600 to 1000 feet for sixteen minutes over a wooded region in Waltham, Massachusetts. The dust counts observed on that date were also higher than have been experienced in any other flight.

TYPES OF BACTERIA ISOLATED

Transfers from colonies on plates were made of characteristic micro-organisms which were obtained from collections made at altitudes above 10,000 feet. Bacterial cultures of this origin were subjected to the common dilution plate methods to obtain pure cultures, which were subsequently examined by the biochemical, physiological and morphological means necessary for identification. The following bacteria were identified in accordance with the characteristics cited in Bergey's *Manual of Determinative Bacteriology*, 4th edition, 1934.

Bacillaceae
Bacillus aerosporus
Bacillus albolactis
Bacillus cereus
Bacillus evanidus
Bacillus Freudenreichii
Bacillus ruminatus
Bacillus simplex
Bacillus teres
Bacillus vulgatus

Bacteriaceae
Achromobacter gasoformans
Achromobacter Pikowskyi
Achromobacter reticularum
Achromobacter stoloniferum
Kurthia Zopfii

Coccaceae
Staphylococcus albus
Staphylococcus citreus
Micrococcus candidans
Micrococcus flavus
Micrococcus ureae
Micrococcus varians

TYPES OF MOLDS ISOLATED

A number of characteristic mold colonies obtained above 10-000 feet were examined morphologically after transfer and cultivation on Czapek's agar.

Aspergilli and Penicillia were the more common molds found; 12 cultures of the former and 6 of the latter were obtained from these collections.

Molds of the following genera have also been identified from this series of flights; *Rhizopus, Mucor, Oöspora, Monosporium, Macrosporium, Tilachlidium, Fusarium.*

Actinomyces

Seven cultures of *Actinomyces* were isolated in the same collections.

No yeasts were found, although in certain flights previous to this group, yeasts had been obtained.

Pollen

Specimens of pollen were obtained in 3 flights, one of which was at an altitude of over 20,000 feet.

Dust

The dust counts show rather wide variations on various flights and at different altitudes. It is worthy of note that the dust counts are of much greater magnitude than those obtained for micro-organisms. Little is known concerning the origin of this dust, although the logical assumption is that it comes from the

earth. A statistical analysis was made of the dust counts obtained on various days of the week in order to make comparisons of those collections above 10,000 feet with others taken below that level, to observe any possible relation between industrial activity and dust counts. No definite relations of this nature have been determined as yet.

The dust counts on the controls are due to the fact that the disks used as collectors are not free of dust in spite of the fact that they are washed with chloroform previous to use. More extensive preliminary washing after the first few flights was found to lower the control counts in this series.

Precautions were taken to insure the fact no dust was getting into the collector from the exhaust of the airplane motor and are cited in a previous report (Proctor, 1934).

DISCUSSION

The results of these observations concerning the microbiology of the upper air, as shown by the collections made, indicate a variable population, both from the standpoint of bacterial and mold types and numbers. The ability of these living micro-organisms to attain altitudes of 20,000 feet or more through the chance action of air currents is particularly significant as it suggests the almost limitless possibilities of travel in a horizontal direction. The survival of such forms despite the many influences which are unfavorable to their existence is also significant in view of the length of time for which they may remain viable.

Those micro-organisms which have been identified to date are largely types associated with soil, air and water. Some of the molds found were members of genera which contain plant pathogens but no recognized pathogens have been identified as yet. There is no valid reason to believe that such micro-organisms are not subject to equally extensive flights, however.

The presence of pollen at high altitudes also indicates the importance of air as a vehicle for the transmission of wind-borne pollens over wide areas.

The high dust counts obtained in comparison to the numbers of micro-organisms is interesting in view of the various possible

sources of dust, some of which, as from soil, might be also associated with high bacterial counts, while in other cases particles from smoke stacks and industries might be sterile. An investigation particularly directed towards the origin and composition of such particles has recently been initiated.

CONCLUSIONS

The upper air, which is constantly changing with respect to many physical factors, is at the same time a vehicle for the dispersion and transmission of many types of micro-organisms, pollens and dust particles.

The extent of vertical movement of such organisms has been demonstrated to be at least several miles.

The types and numbers of micro-organisms found, which were largely types associated with soil and water, are subject to wide variation. These variations are due to numerous influences which are constantly interacting in the upper air strata, to the varied sources of the micro-organisms and to the ability of these micro-organisms to survive unfavorable conditions.

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AN INVESTIGATION OF THE STERILITY OF FISH TISSUES[1]

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The bacteriological examination of fish tissues to determine whether or not bacteria are present has concerned numerous investigators for over three decades. Müller (1903) reported that the flesh of perch and carp was sterile. Ulrich (1906) concluded that bacteria were present in each of the 40 cod and sole studied by him. The same results were reported in respect to perch, pike, dace, salmon, tench, carp and barb. Bruns (1908), working with fresh, salted, dried and smoked fish, found no bacteria in deep tissues but occasionally found them in surface samples of flesh.

Browne (1918) concluded that normal fish muscle was sterile. Hunter (1920), from an examination of various species of Pacific salmon, stated that flesh from the belly or back of these fish was sterile if taken within two hours after the fish was removed from the water. He also found the blood of fresh salmon to be sterile.

Harrison, Perry and Smith (1926) reported that the flesh of haddock was sterile when examined within three hours after the fish were caught.

Fellers (1926) examined the flesh of Pacific salmon and was unable to obtain bacteria from the muscle of those freshly caught. He also found that bacteria were usually absent from the stomach and intestines of those salmon well advanced in their spawning migration.

Stewart (1930) made 143 cultures from codlings. Bacteria

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were obtained in only 5 of these cultures. These organisms were found to be similar to those occurring in slime on fish and were therefore regarded as contaminants.

Gee (1927) (1930) reported the presence of bacteria in fresh fish muscle. In his earlier report he was consistently able to isolate a spore-forming organism from the flesh of both freshly caught and spoiled haddock. In a later paper he cited the presence of bacteria in the flesh of certain freshly caught haddock, cod and dogfish.

The literature cited would seem to favor the opinion that the flesh of fresh fish is sterile, although conflicting evidence is presented in respect to haddock. In view of the fact that haddock is the most important food fish taken in New England, further information seemed desirable.

The present investigation is concerned primarily with the examination of haddock (*Melanogrammus aeglifinus*). The fish used were, as soon as removed from the water, placed at such a temperature that they were solidly frozen within an hour and were kept below −17.8°C., until examined one to eight weeks later.

SECTION I

The first experiments were conducted on 3 small haddock and 3 large haddock.

Areas were first marked off on the skin of a still frozen fish with a sterile scalpel. The areas laid out on the 3 large haddock were (a) in the anterior region just back of the head, (b) the middle lateral portion, and (c) the caudal region. The same was done on both sides of the fish. A total of 90 samples was taken.

The smaller haddock were of such a size (2 pounds) that samples could be conveniently taken only from the areas in the middle region on both sides of each fish. The number of muscle samples taken from these haddock was 30.

The skin covering these areas was pulled off with pliers. A flap of the underlying surface muscle was then cut away and discarded. A sample of the sub-surface muscle weighing approximately 5 grams was then removed with a sterile metal gouge.

Various dilutions were made from these muscle samples after they had been disintegrated by shaking in sterile water.

From the same areas of the fish, samples of approximately one gram in weight were removed in an identical manner and placed in sterile Petri dishes and in sterile broth tubes. Four 1 gram samples were taken from each area and used as inocula for two plates and two tubes of 0 dilution.

Dilutions

Dilutions (from 1:10 to 1:1,000,000) were used to make duplicate agar plates and duplicate broth tubes.

Culture media

Fish broth and fish agar were used as culture media. Five hundred grams of haddock muscle were ground in a meat grinder and extracted for one hour with 1000 cc. of tap water. This mixture was boiled for five minutes, then filtered and autoclaved to precipitate some of the protein. After filtering a second time, the resulting solution was tubed and sterilized to be used as fish broth. Solid media was made by adding 1.5 per cent of agar to the broth.

Plain nutrient broth and nutrient agar were used in the experiments reported in sections II and III.

Incubation period

All cultures were incubated for one hundred and sixty-eight hours at 20°C., before observations were made to determine the presence or absence of bacterial growth.

Results

The bacteriological results obtained from the examination of 90 samples of muscle excised from 3 large haddock and 30 muscle samples from 3 small haddock may be summarized as follows: A total of 252 plates of various dilutions were made from the muscle of the three large haddock; 246 of these plates were sterile. Of the 6 plates showing the presence of bacteria, 5 contained only single bacterial colonies. The remaining plate showed the pres-

ence of only four colonies. Two hundred and fifty-one broth cultures of muscle in various dilutions, made from the same muscle samples, were found to be sterile after incubation. One broth culture was found to contain bacteria.

Of 126 control plates poured simultaneously, all except 2 plates were sterile after incubation.

Eighty-four plates and 84 tube cultures were made from various dilutions of 30 muscle samples excised from 3 small haddock. After incubation all were found to be sterile. All of 42 control plates were also found to be sterile.

Numerous samples of tissue from the gills, skin and intestines of the 6 haddock showed the presence of bacteria when plated in fish agar after incubation for a week at 20°C.

SECTION II

A second series of bacteriological examinations was made on muscle samples from a group of 8 haddock obtained and stored as described above. As these fish were also to be used for certain bio-chemical studies to be discussed in a later paper, it was necessary to modify the technique used in section I.

Muscle samples of approximately 15 grams were removed from the head, center and tail regions, using aseptic technique. These samples, in sterile containers, were placed in refrigerators, maintained at constant temperatures of 0°, 5°, 10°, 15° and 17°C. All samples, usually 5 in number, from the same fish were incubated at the same temperature. At the end of each 24-hour period one of the 15-gram samples from each fish was removed from storage.

Portions of approximately one gram were cut from each sample at the time that it was taken from the refrigerator. These portions were transferred to sterile nutrient broth tubes. These tubes were incubated at 25°C., and examined for evidence of bacterial growth after incubation. Those showing growth or appearing doubtful were checked by transferring 1 cc. portions to Petri plates, pouring, and examining after suitable incubation.

Of 38 muscle samples examined, only four showed growth, and of these not more than one came from any single fish. The

procedure used in this experiment involved more handling of the samples and therefore more possibility of accidental contamination than was the case in section I.

Transfers of tissue samples from the gills, intestines and skin of this group of haddock showed that all samples made therefrom contained bacteria capable of growing on nutrient agar incubated at 25°C.

SECTION III

Bacteriological examinations were made of the blood from three living fish, two silver hake (*Merluccius bilinearis*), and one squirrel hake (*Urophycis chuss*). The fish were caught off Gloucester harbor. As soon as the fish were taken from the water the ventral portion was wiped with a cloth and 95 per cent alcohol poured over the surface. The alcohol was burned off and a ventral incision was made from the anus to the gills with a pair of sterile scissors. A sterile hypodermic needle was inserted into the beating heart and 0.5 cc. of blood withdrawn. The blood was transferred to a tube containing 4.5 cc. of sterile physiological salt solution.

Several hours later the blood samples in saline were diluted in the laboratory and the various dilutions transferred in 1 cc. amounts to nutrient agar plates and nutrient broth tubes. Half of the cultures were incubated under anaerobic conditions. All were incubated at 25°C., for ninety-six hours.

Due to the small quantities of blood obtained, no dilutions lower than 1:10 were possible. All of the samples from two of the fish were found to be sterile, under both aerobic and anaerobic conditions. Only 1 of the 12 aerobic plates in which blood from the third fish was cultured showed evidence of bacterial growth; the other 11 plates were sterile. This plate had but one bacterial colony.

SUMMARY

One hundred twenty sub-surface muscle samples from 6 different haddock were excised under aseptic conditions and subjected to bacteriological examination. From these samples, 336

aerobic fish-agar plates and 336 aerobic fish-broth tubes were made. Bacteria were found in 6 of the 336 plates, or 1.8 per cent of the plates poured. All of the plates found to contain bacteria were dilution plates and in only one instance were duplicate plates from the same sample. Bacteria were found in 2 of the 126 control plates. Bacteria were found in only one of the 336 tube cultures made from the same samples.

From 38 muscle samples taken aseptically from 8 additional haddock, broth tube transfers were sterile from 34 of the samples and 4 contained bacteria. The methods used in this experiment were such that the possibilities of accidental contamination were more numerous than in the previous experiment.

Evidence was also obtained that the skin, gills and intestines of all the above-mentioned haddock contained bacteria.

Blood removed from the hearts of 3 hake immediately after taking from the water was found to be sterile in all anaerobic and aerobic cultures made from 2 fish. The blood of the third fish was found sterile except for the presence of a single colony on one of the 12 aerobic plates, which was probably an accidental contamination.

The results obtained confirm the findings of Harrison, Perry and Smith (1926), who reported that bacteria were not found in the muscle tissue of freshly-caught haddock, and are in accord with others who have found the flesh of fish to be sterile.

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SOME CHEMICAL CHANGES EXHIBITED IN STERILE AND IN CONTAMINATED HADDOCK MUSCLE STORED AT DIFFERENT TEMPERATURES[1]

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The bio-chemical changes which occur in fish tissues are due to two factors, autolysis and bacterial action. As early as 1903, Müller reported that changes due to autolysis took place when sterile fish tissues were incubated. More recently attention has been focussed on the relative importance of these two causes of bio-chemical change.

Reed, Rice and Sinclair (1929) presented evidence that autolysis accounted for only a small part of the actual decomposition of haddock muscle, although autolysis was found to be an important factor in the decomposition of lobster and clam muscle. They also cited increases in pH of haddock muscle from 6.3 to 7 during the first twenty-four hours of incubation at room temperature, due to bacterial action.

Gibbons and Reed (1930) made certain bio-chemical determinations on incubated sterile haddock muscle tissues which they inoculated with known types of bacteria, and observed that the introduction of bacteria caused an increase in protein decomposition products, such as ammonia and non-coagulable nitrogen.

Hess (1932) reported an investigation of changes in haddock muscle and concluded that only slight autolytic changes took place in haddock muscle tissues held at temperatures between 30° and 36°F. Bacterial decomposition was found to be about twice as great at 36°F., as at 30°F.

[1] Contribution No. 61 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

A number of the investigations concerned with autolytic and bacterial changes in haddock muscle have been conducted on tissue which was ground or macerated previous to incubation, after which bio-chemical determinations were made. The results obtained after storage of ground muscle suspensions may not be strictly representative of conditions which occur in normal muscle tissues not subjected to such treatment. Toluene has been used by some investigators for the purpose of inhibiting bacterial action and thereby eliminating biochemical changes due to bacteria, in order to study autolytic changes. The use of toluene for this purpose may offer certain disadvantages as indicated by the work of Wells and Corper (1912) and of Parker (1928).

In the present investigation a procedure was used which avoided the use of toluene as a bacterial inhibitant and also eliminated the grinding or maceration of muscle samples previous to incubation or storage. This procedure was adopted with a view to clarifying certain features concerning the changes which occur in muscle tissues of haddock due to autolysis and those due to bacterial action.

Haddock were used in these experiments. When taken from the ocean the fish were placed in a cabinet refrigerated with solid carbon dioxide and were frozen within an hour. They were kept at −17.8°C., or below, until examined.

One side of each fish was used as a source of sterile sub-surface muscle samples. The other side was later used as a source of "contaminated" muscle samples. The term "contaminated" is used to designate those samples which remained intact in the fish during a period of storage at various temperatures above freezing. Contaminated muscle therefore would be subject to chemical changes due to both autolytic and bacterial action although such samples were not artificially contaminated with bacteria. All of the sterile samples, excised while the fish was still frozen, were placed in sterile flasks plugged with cotton and capped with metal foil. These flasks were stored at constant refrigerator temperatures. Contaminated samples were taken from the other side of the fish after it had been stored for the same time and at

the same temperature as the sterile samples removed previously. Thus it was possible to make chemical comparisons between these two types of samples, sterile and contaminated, obtained from identical fish and stored at the same temperatures.

Eight haddock were examined. Electrometric titrations, (Stansby and Lemon, 1933), and pH determinations, using the quinhydrone method, were made on all of the muscle samples from these fish. Van Slyke amino-nitrogen determinations, (Van Slyke 1911, 1912, 1915), were made on all muscle samples from four of the fish, each of which was stored at a definite temperature, namely 0°, 5°, 10° and 17°C. The other four fish were stored at 0°, 5°, 10° and 15°C. With these fish, Van Slyke determinations were replaced by formol titrations (Harris modification, Harris, 1923–24), and ammonium nitrogen determinations, (Allen and Davidson, 1919). Bacteriological counts were made on the contaminated muscle samples of the latter fish.

The electrometric titration method of Stansby and Lemon (1933), was advocated by its originators as a procedure for the detection of relative freshness in haddock. This method was intended to follow the disappearance of the protein molecule rather than the formation of definite end products. These investigators found that the buffer capacity of a haddock muscle solution decreased as "primary" or autolytic changes occurred in the tissues. An index of the "primary" change was obtained by titrating within a selected pH range in which the buffer capacity of the inorganic constituents of the muscle was at a minimum, namely pH 6.0 to pH 4.28.

As ammonia was assumed by Stansby and Lemon to be one of the principal products formed by bacterial action, an indication of the amount of ammonia formed and hence the amount of "secondary" decomposition was obtained by titrating the fish muscle suspension from its original pH to pH 6.0.

The test therefore consisted in titrating the fish muscle suspension with 0.0165 *N* HCl from its original pH to pH 6.0 and subsequently from pH 6.0 to pH 4.28. The amount of acid, in cubic centimeters, used in the first titration was believed to indicate bacterial decomposition or "secondary" change, and was

called the B value. The amount of acid used in the titration from pH 6.0 to pH 4.28 was called the A value, and was said to be inversely proportional to the amount of "primary" change or protein hydrolysis which had taken place.

DISCUSSION OF RESULTS

Electrometric titrations

The electrometric titration values obtained are indicated in table 1. The results with this method were found to be somewhat irregular with both sterile and contaminated muscle samples.

The A value of sterile samples showed a decrease in three fish, stored at 0°, 5° and 10°C., in a period of five days. On the other hand, sterile samples from three other fish stored at the same temperatures showed slight increases in final A values over those initially obtained. Those sterile samples held at higher temperatures, 15° and 17°C., showed no significant difference in four days between initial and final values.

The A value of contaminated samples showed a decrease in all cases at the end of the storage period, although with one fish held at 17°C., the difference between the initial and the final A value is slight.

The B values obtained on sterile samples from both haddock held at 0°C., and one haddock held at 10°C., showed an increase. B values for the sterile samples of the remaining five haddock decreased during storage.

The B value of contaminated samples decreased in three fish stored at 5°, 10° and 10°C., and increased in the other five.

Working with unfrozen fresh haddock, Stansby and Lemon stated that a B value of more than 8, when the A value is 24 or more, raises suspicion that the fish has at some time been allowed to stand at too high a temperature.

In view of the fact that 6 of the 8 haddock examined in the present investigation were found to have initial A values above 24 and initial B values of 12 or more, and the history of the samples was known to be such that any exposure at high temperature was eliminated, it would seem that such a standard is not applicable to haddock which have been frozen. Stansby and Lemon also

stated that B values of less than 8 indicate haddock in which no marked bacterial action has occurred. In these experiments, sterile muscle samples from 6 of the 8 haddock examined showed

TABLE 1

Electrometric titration values

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	E. T. VALUE	INCUBATION TIME IN HOURS					
				0	24	48	72	96	120
		°C.							
No. 1	Sterile	0	A	32.37	34.85	23.36	24.35	21.34	18.98
	Sterile	0	B	6.11	6.49	7.25	7.14	6.79	8.40
	Contaminated	0	A	32.37	27.85	35.97	17.51	24.03	18.98
	Contaminated	0	B	6.11	5.95	5.72	10.91	11.05	8.40
No. 2	Sterile	5	A	34.44	31.90	23.90*	23.04	26.42	23.90
	Sterile	5	B	7.20	9.20	9.32*	9.03	6.89	9.90
	Contaminated	5	A	34.44	21.47	22.83	22.07	27.00	25.82
	Contaminated	5	B	7.20	8.92	11.00	11.13	11.79	12.08
No. 3	Sterile	10	A	28.38	37.80	26.12	21.48	21.38	23.39
	Sterile	10	B	12.50	10.15	10.90	10.28	8.19	9.05
	Contaminated	10	A	28.38	27.35	19.73	20.50	19.42	20.41
	Contaminated	10	B	12.50	10.80	13.11	11.04	10.55	10.91
No. 4	Sterile	17	A	24.39	22.36	26.02	23.95	24.16	
	Sterile	17	B	12.95	14.50	12.89	12.88	11.61	
	Contaminated	17	A	24.39	20.43	20.02	25.17	23.87	
	Contaminated	17	B	12.95	15.92	13.80	13.63	15.95	
No. 5	Sterile	0	A	24.92	24.30	24.70	24.92	25.86	25.43
	Sterile	0	B	12.58	13.94	13.17	13.30	14.90	14.40
	Contaminated	0	A	24.92	21.15	26.69	23.37	22.34	22.06
	Contaminated	0	B	12.58	12.68	12.92	13.32	14.08	14.82
No. 6	Sterile	5	A	25.97	25.67	26.54*	26.01	26.23	26.30
	Sterile	5	B	13.90	12.60	10.49*	12.60	12.48	12.30
	Contaminated	5	A	25.97	22.88	25.00	22.20	23.09	20.56
	Contaminated	5	B	13.90	12.67	13.00	14.99	14.34	12.95

* Sample possibly not sterile; section cut from it and incubated in broth showed bacterial growth.

TABLE 1—*Concluded*

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	E. T. VALUE	INCUBATION TIME IN HOURS					
				0	24	48	72	96	120
		°C.							
No. 7	Sterile	10	A	25.01	25.99	26.56	24.50	24.61	25.32*
	Sterile	10	B	12.60	11.54	11.14	10.81	10.96	11.50*
	Contaminated	10	A	25.01	23.90	22.80	22.12	23.12	19.86
	Contaminated	10	B	12.60	13.08	11.70	12.34	12.06	11.99
No. 8	Sterile	15	A	26.04	25.30	24.48	26.05*	26.02	
	Sterile	15	B	12.47	13.40	11.00	9.50*	11.86	
	Contaminated	15	A	26.04	24.01	22.62	25.55	21.62	
	Contaminated	15	B	12.47	13.65	13.03	13.35	16.65	

B values always above 8, and with three exceptions, B values above 10.

Hydrogen ion concentration

The results of the pH determinations are given in table 2.

The initial pH of sterile haddock muscle samples varied from 6.58 to 6.90. Excepting those stored at 0°C., sterile samples showed a general tendency to become more alkaline during the storage period although in several cases there was very little change. Several exhibited slight increases (numerical) followed by a subsequent decrease.

Contaminated samples, with one exception, exhibited slight increases in pH during the course of the experiment.

Van Slyke determinations

The amino nitrogen values are shown in table 3.

The results of Van Slyke determinations carried out on sterile muscle samples indicate a slight increase in amino acids followed by a decrease. Those stored at higher temperatures displayed the initial increase within a shorter period of time.

Contaminated samples from 3 haddock showed an increase in amino-nitrogen values followed by a slight drop and an ultimate increase so that the final values were above those initially ob-

tained. Samples from 1 haddock, stored at 5°C., showed an increase followed by a final decrease to values below those initially obtained.

TABLE 2

pH values of muscle

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	INCUBATION TIME IN HOURS					
			0	24	48	72	96	120
		°C.						
No. 1	Sterile	0	6.65	6.64	6.64	6.80	6.65	6.77
	Contaminated	0	6.65	6.64	6.64	6.64	6.74	6.65
No. 2	Sterile	5	6.58	6.63	6.65*	6.55	6.55	6.55
	Contaminated	5	6.58	6.65	6.73	6.65	6.65	6.65
No. 3	Sterile	10	6.60	6.60	6.70	6.70	6.55	6.50
	Contaminated	10	6.60	6.65	6.75	6.75	6.70	6.65
No. 4	Sterile	17	6.65	6.68	6.70	6.70	6.60	
	Contaminated	17	6.65	6.68	6.75	6.70	6.80	
No. 5	Sterile	0	6.75	6.90	6.90	6.90	6.90	6.90
	Contaminated	0	6.75	6.90	6.70	6.90	6.90	6.90
No. 6	Sterile	5	6.90	6.85	6.87*	6.80	6.85	6.75
	Contaminated	5	6.90	6.85	6.93	6.95	6.95	6.95
No. 7	Sterile	10	6.80	6.81	6.82	6.85	6.75	6.75*
	Contaminated	10	6.80	6.80	6.83	6.87	6.84	6.95
No. 8	Sterile	15	6.75	6.77	6.75	6.70*	6.74	
	Contaminated	15	6.75	6.76	6.82	6.87	6.95	

* Samples possibly not sterile; sections cut from it and incubated in broth showed bacterial growth.

Formol titrations

The data for formol titrations are given in table 4.

Sterile muscle samples showed a rise in amino acids followed by a decrease. In no case was there a final increase in amino acids.

With contaminated samples there was, in each case, an initial rise in value followed by a decrease and a subsequent increase.

It is interesting to note that at the higher temperatures of storage, 15° and 17°C., amino-acid values obtained in contami-

TABLE 3

Van Slyke determinations

Milligrams of amino nitrogen per gram of muscle

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	INCUBATION TIME IN HOURS					
			0	24	48	72	96	120
		°C.						
No. 1	Sterile	0	0.7437	0.7519	0.7728	0.7311	0.6164	0.6369
	Contaminated	0	0.7437	0.8123	0.7607	0.9779	1.0337	1.1977
No. 2	Sterile	5	0.8109	0.8499	0.7110*	0.9880	0.8643	0.8214
	Contaminated	5	0.8109	0.8445	0.9250	1.0742	0.8525	0.6377
No. 3	Sterile	10	0.7684	0.8897	0.8989	0.8036	0.6947	0.6814
	Contaminated	10	0.7684	0.9347	0.6351	0.8266	0.8303	0.9179
No. 4	Sterile	17	0.5324	0.7425	0.5990	0.5871	0.5891	
	Contaminated	17	0.5324	0.6233	0.6961	0.6586	0.9527	

* Sample possibly not sterile; section cut from it and incubated in broth showed bacterial growth.

TABLE 4

Formol titration values

Cubic centimeters *N*/10 KOH per 10 grams of muscle

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	INCUBATION TIME IN HOURS					
			0	24	48	72	96	120
		°C.						
No. 5	Sterile	0	9.82	10.06	13.00	11.30	11.71	10.53
	Contaminated	0	9.82	9.90	12.01	10.25	11.00	11.90
No. 6	Sterile	5	10.10	10.50	10.10*	10.60	9.15	8.20
	Contaminated	5	10.10	10.60	9.10	10.54	11.09	12.38
No. 7	Sterile	10	10.44	12.31	13.29	12.91	12.06	11.64*
	Contaminated	10	10.44	13.58	11.59	13.27	13.50	13.94
No. 8	Sterile	15	12.22	13.07	14.52	13.37*	12.60	
	Contaminated	15	12.20	13.08	12.46	14.83	16.49	

* Sample possibly not sterile; section cut from it and incubated in broth showed bacterial growth.

nated samples were increased, as shown by the results of both the formol titration and the Van Slyke method. In four days, higher values were reached at these temperatures, from the standpoint of percentage increase, than were obtained in five days at the lower temperatures.

Ammonium nitrogen

The ammonium-nitrogen values obtained on 4 haddock are given in table 5. The values for the sterile samples indicate an increase followed by a very slight decrease. The rate of increase

TABLE 5

Ammonium nitrogen values in milligrams per 100 grams of muscle

HADDOCK	MUSCLE SAMPLE CONDITION	TEMPERATURE OF INCUBATION	INCUBATION TIME IN HOURS					
			0	24	48	72	96	120
		°C.						
No. 6	Sterile	5	0.0054	0.0062	0.0089	0.0094	0.0092	0.0090
	Contaminated	5	0.0054	0.0064	0.0091	0.0098	0.0108	0.0128
No. 7	Sterile	10	0.0060	0.0089	0.0091	0.0085	0.0081	0.0086*
	Contaminated	10	0.0060	0.0087	0.0103	0.0122	0.0176	0.0185
No. 8	Sterile	15	0.0061	0.0110	0.0111	0.0123*	0.0098	
	Contaminated	15	0.0061	0.0117	0.0132	0.0155	0.0247	

* Sample possibly not sterile; section cut from it and incubated in broth showed bacterial growth.

was greater at the higher temperatures of storage and in all cases the final values were well above those initially observed.

Contaminated samples gave increases in ammonium nitrogen values of greater magnitude than those observed in sterile muscle samples; this difference was particularly marked at the 10° and 15°C., storage temperatures.

Bacteriological counts

Bacteriological tests for sterility were carried out on each of the sterile samples used for analyses on the completion of the storage periods. Those samples, isolated aseptically, which

were found to be contaminated, are indicated by an asterisk in the tables.

Bacterial plate counts were conducted on contaminated muscle samples from four fish as shown in table 6. The amount of bacterial infection in each contaminated sample was subject to variation and doubtless differed considerably at the start of the experiment since the sources of such infection are those micro-organisms present on the skin, gills and in the alimentary tract of the different fish when caught.

TABLE 6

Bacterial counts on haddock muscle

HADDOCK	TEMPERATURE OF INCUBATION	BACTERIA PER GRAM OF MUSCLE INCUBATION TIME IN HOURS					
		0	24	48	72	96	120
	°C.						
No. 5.....	0		120	140	500	800	2,900
			130	150	500	900	3,200
No. 6.....	5		800	1,200	3,000	4,200	7,200
			800	1,000	3,800	4,700	8,900
No. 7.....	10		300	550	4,700	98,000	390,000
			325	500	3,800	110,000	460,000
No. 8.....	15		46,000	130,000	210,000	3,100,000	
			52,000	130,000	220,000	3,900,000	

SUMMARY

The results obtained with the electrometric titrations were irregular and did not conform to the limits set by the originators of this method as applicable to unfrozen haddock under specific conditions. The fish used in our experiments had previously been frozen and this fact may account for the difficulties encountered in the interpretation of their standards.

The changes in pH were small in magnitude but there was observed a general tendency toward a higher pH on storage of the contaminated muscle samples, while sterile muscle samples tended to decrease slightly in pH.

Amino-acid values, as obtained by the Van Slyke method and the titration method of Harris, indicated an increase in amino acids in sterile samples followed by a rather definite decrease. Contaminated samples in general showed an increase in amino acids in the later part of the storage period. The increase indicated by contaminated samples in the later part of the incubation period is assumed to be the result of bacterial action. The reason for the final decrease in amino acids in sterile samples is not known. It is possible that some of the bio-chemical changes associated with *rigor mortis* may be concerned, as these fish were kept at sub-freezing temperatures from the time that they were caught until the storage experiment started.

Ammonium nitrogen values indicated that bacterial action in haddock muscle is not extensive during the first twenty-four hours when stored at temperatures as high as 17°C. Thereafter bacterial action becomes increasingly important. It is probable that at temperatures of 5°C., and higher, bacterial action is the chief factor in the decomposition of haddock tissues.

CONCLUSIONS

Changes of a chemical nature took place in sterile haddock muscle at refrigerator temperatures ranging from 0° to 17°C. The extent of such changes differed from those occurring in muscle from the same fish which were subject to bacterial action also. More marked changes occurred in the latter, particularly at the higher temperatures.

Since in commercial haddock fisheries the presence of bacteria in the muscle is inevitable, due to practical operating conditions, the importance of low storage temperatures to limit those chemical changes, which eventually cause spoilage, is indicated.

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A METHOD FOR ESTIMATING THE BACTERIAL CONTENT OF THE MOUTH BY DIRECT COUNT[1]

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An accurate quantitative method of estimating bacteria in the mouth is of great value in following the progress of pathological conditions and the effect of antiseptics as well as the changes due to certain diets. Cultural methods have been unsatisfactory because many different kinds of media under aerobic and anaerobic conditions are necessary in order to cultivate the numerous types of organisms which inhabit the mouth. The danger of out-diluting organisms in attempts to produce suitable plates for counting must also be borne in mind.

The methods of making direct counts have many realized defects. They have, however, given consistently higher values than the plate method. Kligler (1915), in comparing the two methods, found that the microscope count was about five to six times greater than the plate count.

One of the major difficulties in the direct-count method has been the clumping of bacteria, due to mucin and epithelial cells in the saliva and to the thread forms which entangle masses of bacteria. It was with the aim of separating these clumps of bacteria, so as to have a uniform distribution of organisms in a smear, that the following method was worked out. In general, the procedure followed was similar to that used by Breed and Brew (1933) for making direct bacterial counts of milk, with the exception that an atomizer was employed to break up the larger masses and a very dilute sodium hydroxide solution was used as a diluent to dissolve the mucin.

[1] We wish to acknowledge the contributions of Dr. Irwin Steinberg and Faith P. Hadley to the first stages of this study.

DESCRIPTION OF ATOMIZER

A large test tube about 3.5 cm. in diameter and 17 cm. in length was used (see figure 1). This test tube had a side arm about 6 cm. from the top for the entrance of compressed air. A bulb about 3 cm. in diameter, opposite to this side arm, joined the

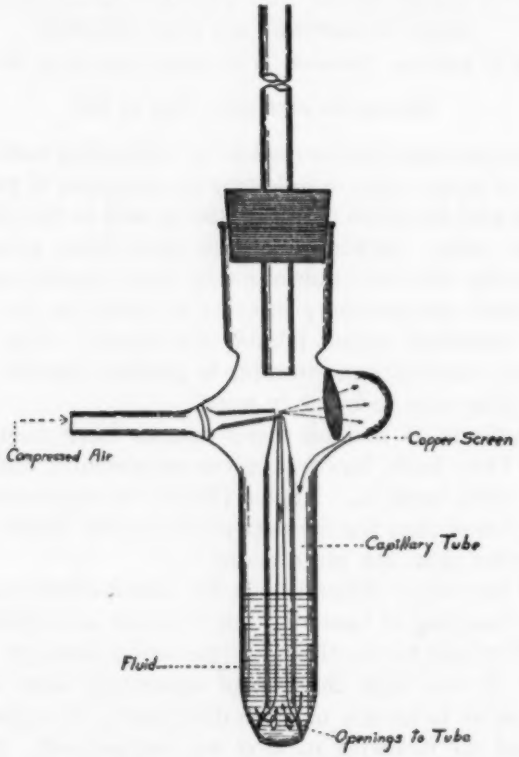

FIG. 1. DIAGRAMMATICAL DRAWING OF ATOMIZER (ARROWS SHOW PATH OF FLUID)

main tube at an angle of about 60°. The bulb was separated from the main tube by a piece of 100-mesh copper screening fused to the walls of the tube. A capillary tube with perforations at its base was sealed to the bottom of the test tube. This was fixed so as to meet at right angles the capillary air-delivery

tube sealed to the side arm. The fluid entered the capillary tube through the perforations at the base and was continuously sprayed through the copper screen by the air stream. A rubber stopper fitted with a piece of glass tubing about 10 cm. long allowed the air to escape and returned spattered fluid to the vessel. During operation of the device the fluid was constantly sprayed and mixed. It was found that spraying for thirty minutes gave the best results.

Since mucin is dissolved by alkali, it was hoped that an alkali strong enough to act on the mucin without disintegrating the bacteria would aid the ease and accuracy of making the counts. We have used $N/400$ sodium hydroxide. Doubtless other alkalies such as sodium carbonate and sodium phosphate would work equally well.

METHOD OF SAMPLING

We found that after a meal the number of bacteria removed from the mouth decreased as much as 78 per cent. To eliminate this factor, only liquids which could be taken through a drinking tube were consumed during an eight-hour period. The subjects refrained from smoking and had breakfast at least two hours before the first sample was collected. Teeth were not previously brushed in the morning. Samples were also obtained three and six hours after the first preparation.

Samples were collected by thoroughly brushing the teeth, care being taken that the same procedure of brushing was followed each time by each subject. (Uniform tooth brushes were used.) After the brushing, the mouth was vigorously rinsed with 10 cc. of distilled water and this transferred, together with washings from the tooth brush, to the atomizer; and the washings were sprayed for thirty minutes. The volume was then made up to 20 cc. with distilled water (4 to 7 cc. were lost by evaporation during the period of spraying).

A capillary pipette calibrated to deliver 0.01 cc. was used for sampling the washings. Slides were used having etched lines forming two areas of one centimeter square.

The washings were thoroughly mixed and 0.01 cc. deposited

on each of the areas. The samples were spread evenly over the square centimeter area by means of a stiff straight platinum needle. The slides were allowed to dry on a level surface protected from dust. The films were then Gram-stained.

Fields free of epithelial cells and clumps were selected for counting. While clumps of bacteria were not entirely done away with by this method, they were decidedly less numerous and not as dense as in unsprayed material. In some cases where the bacterial count was unusually high, the sample was diluted 1:5 or 1:10.

TABLE 1

Effect of spraying

NAME	TOTAL NUMBER OF BACTERIA		INCREASE
	Before spraying	After spraying	
			per cent
W (1)	2,210,000,000	3,500,000,000	36
W (2)	2,060,000,000	4,720,000,000	36
C	2,630,000,000	4,160,000,000	60

TABLE 2

Comparison of counts using distilled water and N/400 sodium hydroxide

NAME	DISTILLED WATER	*N*/400 NaOH	INCREASE
			per cent
C	3,220,000,000	3,780,000,000	14
H	2,370,000,000	2,440,000,000	0.02
K	3,830,000,000	3,850,000,000	0.9

The bacteria in four ocular fields of each square were counted. The number of bacteria was calculated from the average. The microscope was adjusted so that the factor 8,264 was obtained (area of square on slide 100 sq.mm. divided by area of ocular field, 0.0121 sq.mm.) to give the number of bacteria in 0.01 cc. of washings. Multiplication by 2000 gave the number of bacteria removed from the mouth in the 20 cc. volume. The difference between counts of duplicate preparations from each of 34 samples averaged 13 per cent, the maximum being 31 per cent and the minimum, 1 per cent.

A comparison of smears made fron the same mouth washings

before and after spraying shows conclusively the advantages of the spraying. Three separate counts in preparations from two

TABLE 3

Variations in counts

INDIVIDUAL	TOTAL NUMBER OF BACTERIA REMOVED FROM MOUTH ON DIFFERENT DAYS		
	Initial count	3 hours later	6 hours later
F. H.	2,710,000,000	4,130,000,000	3,700,000,000
	3,910,000,000	3,170,000,000	4,850,000,000
	3,960,000,000	3,530,000,000	3,810,000,000
	5,140,000,000	5,860,000,000	5,930,000,000
	11,220,000,000	7,070,000,000	7,320,000,000
	4,690,000,000	4,760,000,000	7,050,000,000
M. K.	2,620,000,000	2,060,000,000	3,250,000,000
	3,370,000,000	2,950,000,000	2,920,000,000
	3,660,000,000	2,240,000,000	3,000,000,000
	3,730,000,000	2,470,000,000	2,850,000,000
	5,090,000,000	4,800,000,000	4,740,000,000
M. C.	4,130,000,000	3,700,000,000	3,780,000,000
	6,920,000,000	2,660,000,000	4,240,000,000
	5,250,000,000	3,400,000,000	2,440,000,000
	4,880,000,000	3,650,000,000	3,860,000,000
	2,540,000,000	3,230,000,000	2,940,000,000
	4,160,000,000	4,770,000,000	3,730,000,000
	5,850,000,000	4,060,000,000	3,960,000,000
	2,540,000,000	4,760,000,000	5,850,000,000

TABLE 4

Average variation in counts expressed in per cent

NAME	NUMBER OF COUNTS AVERAGED	3 HOURS LATER	6 HOURS LATER
		per cent	*per cent*
F. H.	6	−10	+3
M. K.	5	−21	−9
M. C.	8	−12	−10
O. Z.	3	+27	+21
R. F.	5	−6	−0.7

subjects show an increase of 36 to 60 per cent in the total numbers after atomizing (table 1).

When the sodium hydroxide was used, the procedure was as

follows. Mouth washings were taken as described above by brushing the teeth and rinsing the mouth with distilled water. The amount of washings was measured and divided into two equal parts. One part was made up to 20 cc. with distilled water, the other made up to the same volume with $N/400$ sodium hydroxide. Both were sprayed for thirty minutes and again made up to volume (20 cc.) with either sodium hydroxide or distilled water as before. The sodium hydroxide treated portions gave a better distribution and the fields were easier to count, although some clumps were still present and the counts were not much higher. Table 2 shows counts obtained by this procedure.

The variations in the counts from the same individual at the same time of day, but on different days are illustrated in table 3.

Feirer and Leonard (1931) report a high percentage increase of bacteria in mouth washings over a period of seven hours. Our results do not agree with this. Table 4 shows the average percentage variations from the first count, and indicates individual variations.

SUMMARY

1. This method differs from other methods mainly in the use of an atomizer which breaks up the larger masses of bacteria in mouth washings. The percentage difference in counts made by this method is 13 per cent.

2. The number of bacteria in sprayed washings increased appreciably (at least 36 per cent) over the unsprayed material.

3. $N/400$ sodium hydroxide treated washings gave a better distribution of bacteria in a smear without, however, materially increasing the count.

4. Counts taken at different times of the day and on different days varied greatly in the same individual.

5. No correlation was noted between counts made by the method described and the "diurnal tide" of Feirer and Leonard.

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THE DENATURATION OF STAPHYLOCOCCAL PROTEINS[1]

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Bacteriologists employ the term "denaturation" in a loose sense to imply alteration of antigenic complexes without defining clearly the nature or extent of these changes. It is questionable whether such use of the term is justified, for from the biochemists' viewpoint "denaturation" has a specific chemical connotation and refers to an intramolecular rearrangement of the protein molecule evidenced by certain altered physical and chemical properties which in turn may lead to gross changes in state. Proteins in general become insoluble at the iso-electric point when denatured (Hardy, 1899); they lose their capacity for crystallization; their solutions show a large increase in viscosity (Anson and Mirsky, 1932), a rise in specific optical rotation (Young, 1922) and sulphydryl or disulphide groups undetectable in the native protein can be demonstrated by means of color tests and oxidation-reduction reactions (Arnold, 1910; Harris, 1923; Walker, 1925). As compared to other chemical reactions, protein denaturation has a characteristically high temperature coefficient, a rise of 10°C. increasing the rate of reaction several hundred times (Chick and Martin, 1910, 1911). This characteristic offers a basis for inferring that such processes as the inactivation of enzymes and the killing of bacteria by heat involve basically a protein denaturation since they too possess extraordinarily high $Q_{10°s}$. Proof of this hypothesis, more satisfactory than analogy, is available in the case of pepsin, for when it is heated the loss of activity is paralleled quantitatively by its denaturation (Northrop 1930).

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In an earlier publication one of us outlined a method for the preparation of bacterial antigens (Krueger, 1933) designed particularly to avoid denaturation of the cellular proteins. Briefly the technic consisted of the following steps: 1. Growth of mass cultures. 2. Washing of organism in Locke's solution to remove metabolities. 3. Fragmentation of cells in a special ball mill. 4. Filtration of the ground suspension through acetic-collodion ultrafilters. 5. Standardization of the sterile filtrate on the basis of native protein content.

Clinical experience with such undenatured antigens has indicated that they have a considerable range of therapeutic and prophylactic application (Frawley, Stallings and Nichols, 1934; Frawley, 1934; Stallings and Nichols, 1934; Munns and Aldrich, 1934; Kracaw, 1934; also unpublished work by Stallings and Nichols, Kracaw and Hosmer) and that their use is attended by a minimum of non-specific reactions on the part of the patient. While our preparations contain known concentrations of native proteins we were hampered by lack of specific information as to their properties and could not state except in a very general way just how the antigens should be handled to avoid denaturation. Of particular practical importance was the question of temperature effects, not only for defining the limits within which the proteins could be maintained in the native form but also for demonstrating experimentally how an antigen made by this special technic differs from a heat-killed vaccine. The present paper is a record of experimental work on the heat denaturation of staphylococcal proteins.

EXPERIMENTAL

Fairly concentrated preparations of UBA (undenatured bacterial antigens) were made according to the method of Krueger (1933) using several strains of *Staphylococcus aureus*. They all gave the usual qualitative tests for protein and strongly positive Molisch tests. Direct determinations were made of total protein nitrogen and the nitrogen associated with the component native and denatured protein fractions. In all cases the denatured protein fraction was between 2 and 10 per cent of the total pro-

tein present. The solutions were exposed to various temperatures in a constant temperature bath; samples were taken at intervals and the denatured protein nitrogen, the native protein nitrogen and the non-protein nitrogen content determined as outlined under "methods." In this way it was possible to follow the course of the denaturation reaction in protein preparations representing the naturally occurring compounds of the bacterium, unaltered except as they may have been modified by rupture of the cell membrane and mechanical agitation.

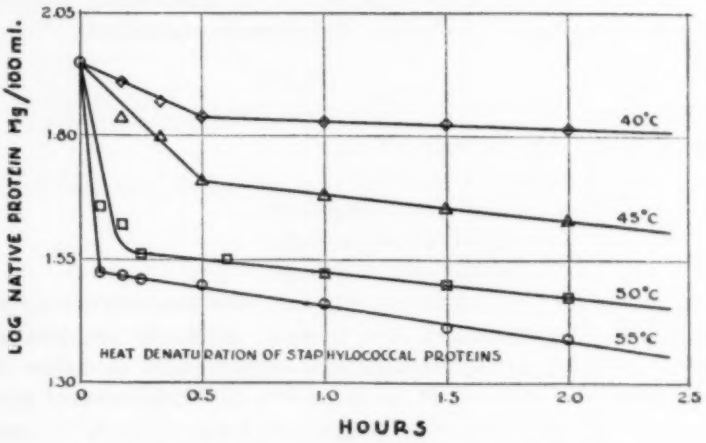

FIG. 1. HEAT DENATURATION OF STAPHYLOCOCCAL PROTEINS

Total nitrogen content = 41.5 mgm. per 100 cc. Total protein content = 89.0 mgm. per 100 cc. Denatured protein content = 7.55 mgm. per 100 cc. Native protein content = 81.45 mgm. per 100 cc.

In figure 1 are shown the denaturation curves for 40°, 45°, 50°, and 55°C. on one particular preparation made from a single strain of staphylococcus known as S-3-k, plotted as logarithms of residual native protein in milligrams per 100 ml. against time of exposure. For each temperature there was apparently a primary rapid denaturation reaction followed by a second slower denaturation. Considering only the first or rapid phase of denaturation at each temperature the curves follow the course of a monomolec-

ular reaction. The velocity constants calculated from $k = \frac{1}{t} ln \frac{P_o}{P_o - P_D}$ for these portions of the curves are:

40°C.	45°C.	50°C.	55°C.
$k = 0.00806$	$k = 0.0181$	$k = 0.064$	$k = 0.198$

where: k = velocity constant
t = time of exposure
P_o = original native protein per millimeter
P_D = protein denatured in time (t).

The van't Hoff-Arrhenius equation for the relationship between rate of reaction and the absolute temperature states that:

$$k_2 = k_1 e^{\mu/2 \left(\frac{T_2 - T_1}{T_2 T_1}\right)}$$

where: k_2 = velocity constant at T_2
k_1 = velocity constant at T_1
T_2 = second absolute temperature
T_1 = first absolute temperature
μ = critical thermal increment.

As a test for the constancy of μ over the temperature range studied the logarithms of the velocity constants are plotted against reciprocals of the absolute temperature in figure 2. Apparently μ for the heat denaturation of staphylococcal proteins is constant within the temperature range 40 to 55°C. and has a value of about 44,000.

In addition to the development of insolubility at the iso-electric point another property distinguishing denatured proteins is the appearance of sulphydryl groups. Our solutions of staphylococcal proteins as originally prepared gave no color reaction for SH groups, but after heating the nitroprusside reaction of Harris (1923) became strongly positive. In figure 3 the logarithms of sulphur in the SH form expressed as mg. of sulphur per milliliter are plotted against the time of exposure to heat; the SH groups appear rapidly as the protein solution is heated.

Other preparations of staphylococcal proteins from various strains of Staphylococci have given results, in general, comparable to those noted above for one particular preparation.

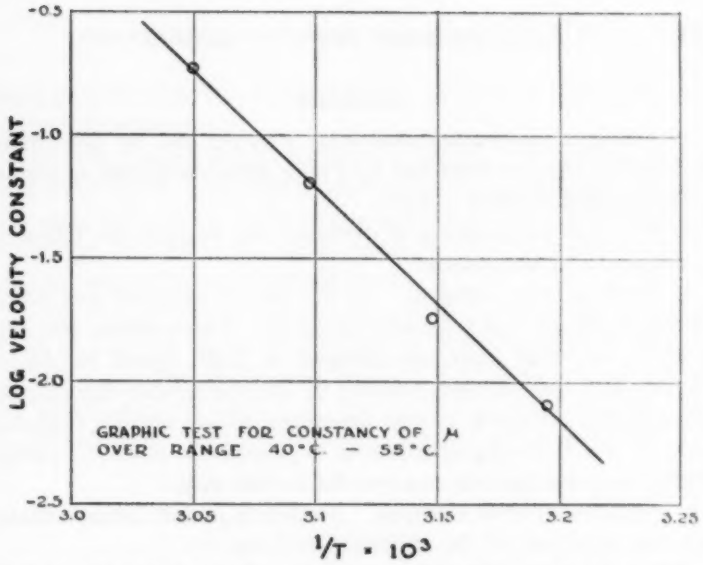

FIG. 2. GRAPHIC TEST FOR CONSTANCY OF CRITICAL THERMAL INCREMENT OVER TEMPERATURE RANGE STUDIED

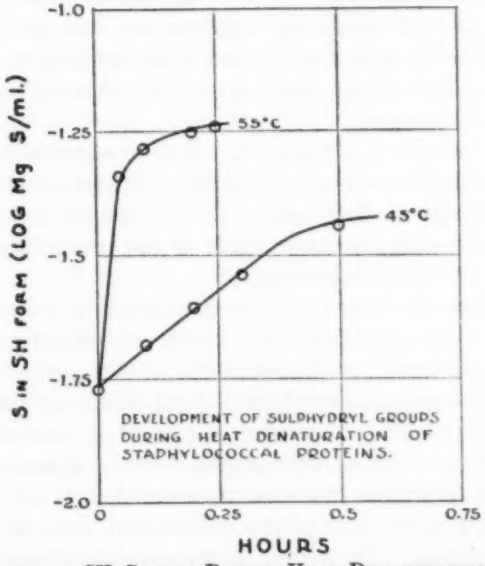

FIG. 3. TITRATION OF SH GROUPS DURING HEAT DENATURATION OF STAPHYLOCOCCAL PROTEINS

METHODS

1. Nitrogen determinations were carried out by the micro-Kjeldahl method as described by Pregl (1930) using the apparatus of Parnas and Wagner (1921).

2. For each preparation of undenatured antigen the following determinations were made:

a. Total protein nitrogen. To 5.0 ml. of solution was added 5.0 ml. of 10 per cent trichloracetic acid. The solution was kept at 20°C. one-half hour, centrifuged at high speed for fifteen minutes and the nitrogen content of the precipitate determined. Throughout our work it was necessary to use special digestion flasks in which the small amounts of precipitate could be packed solidly; loss of materials was avoided in this way.

b. Total non-protein nitrogen. A direct Kjeldahl determination was run on 5.0 ml. of the supernatant from (*a*).

c. Denatured protein. One milliliter of citrate buffer (Sørensen's citrate-HCl mixture) pH 4.6 was added to 4.0 ml. of solution and the mixture was kept at 20°C. for ten minutes. After centrifugation at high speed the supernatant was poured off into another digestion flask for (*d*) and a determination run on the precipitate. This latter figure gave the nitrogen equivalent of the denatured protein.

d. Native protein determination. To the supernatant from (*c*) there was added 5.0 ml. of 10 per cent trichloracetic acid. The mixture was kept one-half hour at 20°C. and was then centrifuged. A Kjeldahl determination was made on the precipitate giving the native protein nitrogen content.

3. To follow the course of the denaturation reaction 4.0 ml. aliquots of antigen were placed in the special digestion tubes and exposed to 40°, 45°, 50°, and 55°C. in the water bath. At intervals tubes were removed and 1.0 ml. of citrate buffer pH 4.6 added to the antigen. The tubes were held at room temperature ten minutes and then centrifuged. The supernatants were poured off into another digestion tube and a Kjeldahl determination was made on the precipitate. This gave a direct measure of denatured protein. To 5.0 ml. of supernatant there was added 5.0 ml. of 10 per cent trichloracetic acid. After centrifugation a determination was run on the precipitate and on the supernatant

giving respectively the residual native protein nitrogen and the non-protein nitrogen.

With the above data available for each preparation it was possible to check back and to determine the percentage error at each step and also to ascertain whether protein hydrolysis was occurring. In the experiments cited the loss of protein by hydrolytic cleavage was extremely small.

4. For the quantitative determination of SH groups standard iodine in KI was added to aliquots of the test solution and the residual iodine remaining after reaction with available SH groups was determined by back titration with standard sodium thiosulfate solution. The general equation for the reaction is:

$$2\,RSH + I_2 \longrightarrow R - S - S - R + 2\,HI$$

DISCUSSION

The experimental data on the course of the heat denaturation of staphylococcal proteins indicate that there is first a rapid denaturation followed by a slower denaturation for each of the temperatures studied. The reactions proceed logarithmically with time and have a critical thermal increment of the same order of magnitude as the values reported for the spontaneous destruction of a variety of proteins. For example, Chick and Martin (1910, 1911) studied the heat denaturation of hemoglobin in distilled water over the range 60° to 70°C. and found the temperature coefficient to be 13 corresponding to a μ of about 55,000. Our preparations of staphylococcal proteins gave an average of μ = 44,000. This difference in magnitude of μ is not serious and may be attributed to the natural variation between different proteins and the effects of the conditions under which the experiments are performed. Denaturation as a rule proceeds most rapidly at the iso-electric point (Chick and Martin, 1910, 1911) and is inhibited by the presence of salts (Handovsky, 1910). The conditions obtaining in our work were not optimal, for the pH was 7.2 (iso-electric point = 4.6) and the solvent employed was Locke's solution. We found that runs made with solutions adjusted to pH 4.6 showed material increases in velocity, μ increasing as high as 53,000.

If the value $\mu = 44{,}000$ applies to the additional 5° increment between 55° and 60°C. as it does to the 40° to 55°C. range for which it has been shown to hold, then $k = 0.54$ at 60°C. Using this velocity constant in the equation for the velocity of a monomolecular reaction to calculate the amount of native protein nitrogen left after four minutes' exposure to a temperature of 60°C. it is found to be 1.61 mgm. or less than 12 per cent of the original amount present.

Our data then suggest that at the temperature ordinarily employed for killing bacteria in the preparation of vaccines, i.e., 60°C., substantially all of the staphylococcal proteins are denatured. It is possible to avoid this denaturation to a very considerable degree by substituting mechanical fragmentation of the cells for heat treatment as outlined in a previous publication (Krueger, 1933). Further, it would appear that undenatured bacterial antigens made according to this technic may be maintained at ordinary room temperature without danger of denaturation. Temperatures of 40°C. and over will produce rapid denaturation of the constituent proteins. It is recognized that our studies deal with rather crude solutions and, purposely, no attempt has been made to purify them in order that our data might be interpreted as applying to the preparations used for clinical work.

SUMMARY AND CONCLUSIONS

1. Staphylococcal antigens made by mechanical fragmentation and ultrafiltration according to the method of Krueger, contain less than 10 per cent of the total bacterial protein in the denatured form.

2. On exposure to heat, staphylococcal native proteins are denatured, becoming insoluble at the iso-electric point (pH 4.6) and showing a considerable increase in SH groups. The denaturation reaction obeys the Mass Law and has a critical thermal increment averaging about 44,000.

3. These data have practical implications in regard to the heat killing of bacteria for vaccines and also with respect to the conditions necessary for preservation of undenatured bacterial antigens.

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ULTRAFILTRATION EXPERIMENTS WITH THE VIRUSES OF LARYNGOTRACHEITIS AND CORYZA OF CHICKENS[1]

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Ultrafiltration experiments with laryngotracheitis virus were first reported by Beach (1930), Gibbs (1931) and Beach (1931). These investigators used the porcelain filters of Berkefeld and the fibrous filters of Seitz and agreed that the virus particles passed readily through the Berkefeld V filters, occasionally through the Berkefeld N filters, and were completely retained by the Berkefeld W filters using water pressure systems. There was some disagreement over the Seitz filtration experiments. Beach did not find sufficient virus in his ultrafiltrates to infect susceptible chickens, while Gibbs thought these filters superior to Berkefeld N filters, but not as consistent in results as the Berkefeld V filters.

Later Gibbs (1933) introduced hormone broth as a menstruum for the suspension of laryngotracheitis virus particles, according to the technique of Ward and Tang (1929), Ward (1929) and Tang (1930), and found some improvement in filtration, but the results were far from satisfactory because the mucous and tissue cells in the exudate were not removed. During centrifugalization some of the virus particles were apparently thrown down with the cellular débris, while finer particles still remained in suspension to plug the pores of the filters.

The purification of suspensions of vaccine virus by adding dilute solutions of organic acids until the iso-electric point is

[1] Contribution number 218, Massachusetts Agricultural Experiment Station.

reached, and titrating back to the neutral point with dilute sodium carbonate, as suggested by Behrens and Morgan (1932) and Behrens and Nielson (1935) suggested a new approach to these filtration studies of infectious laryngotracheitis virus, to which was added later a similar study of coryza.

The iso-electric point of laryngotracheitis exudate was readily determined by adding a 0.04 molar solution of citric acid, drop by drop, to a triturated suspension in distilled water. When the iso-electric point was reached, the cellular débris flocculated and settled to the bottom of the tube, leaving a faintly turbid supernatant suspension of the virus. The settling of the cellular material may be aided by slow centrifugalization, and the supernatant fluid pipetted off and neutralized with a 1/25 molar solution of sodium carbonate, using 0.04 per cent brom-thymol-blue as an indicator. This neutralized supernatant fluid will be referred to in this paper as the purified virus suspension.

Furthermore, it was found that the purified virus suspension passed through the collodion membranes of Cox and Hyde (1932) and Allisbaugh and Hyde (1935 with comparatively little absorption or decrease in virulence. This method of ultrafiltration is a marked improvement over the technique that has been used hitherto for the study of laryngotracheitis virus.

THE ETIOLOGY OF INFECTIOUS CORYZA

Recently Gibbs (1935) has used this method of ultrafiltration in the study of the etiology of infectious coryza or epidemic colds in chickens and found that six widely separated outbreaks in Massachusetts were due to filterable agents. Secondary bacterial infection was found in exudates from four of these outbreaks that were somewhat virulent to susceptible fowls and chickens, but the microörganisms belonged to three different bacterial families and could not have been the causative agent involved in this disease.

The relation of these secondary microörganisms to coryza and laryngotracheitis have been studied in this laboratory for five years, and considerable evidence has been secured to indicate that there are some important secondary invaders in both of these

diseases. The results of these studies on secondary infection in coryza and laryngotracheitis will form the basis of another report. It is sufficient to state in passing that they have been eliminated from these ultrafiltration studies as much as possible.

ULTRAFILTRATION STUDIES

A comparative study was made of the ultrafiltering properties of laryngotracheitis and coryza viruses. These studies revealed for the first time that the virus suspensions before purification and after purification, though diluted 100 times with distilled water, were more virulent than the undiluted fresh exudate from the larynx and trachea of diseased chickens. The reason for this increased virulence is unknown, unless it is due to the fact that

TABLE 1

Virulence of laryngotracheitis virus

	NUMBER OF CHICKS		
	Died	Recovered	Unaffected
Fresh tracheal exudate	40	20	20
Suspension before determining iso-electric point	60	16	4
Neutral suspension after determining iso-electric point	59	17	4
Purified virus suspension	56	18	6

the virus particles are contained in the nuclei of the cells in the fresh exudate, as stated by Seifried (1930), and many of them are destroyed before entering the living cells of the mucous membrane of the inoculated chickens. When the triturated virus suspensions are inoculated intratracheally into susceptible chickens the virus particles are free to enter directly into the living cells of the mucous membrane of the host.

The virulence of laryngotracheitis virus as found in this experiment is recorded in table 1.

Inspection of table 1 will show that the suspension before determining the iso-electric point and the neutral suspension after determining the iso-electric point were more virulent than either the fresh tracheal exudate or the purified virus suspension, and

furthermore that the purified virus suspension was more virulent than the fresh tracheal exudate.

The virulence of the coryza virus used in this experiment is as shown in table 2.

The results in table 2 show that the coryza virus, freed from secondary microörganisms and inoculated into susceptible chickens reared in the laboratory and likewise free from secondary infection, was not as virulent as the laryngotracheitis virus, although the respiratory tract was affected in both cases. The three coryza suspensions were virulent in the same order as the laryngotracheitis suspensions, but not in the same degree.

TABLE 2

Virulence of coryza virus

	NUMBER OF CHICKS		
	Died	Recovered	Unaffected
Fresh nasal exudate	0	20	60
Suspension before determining iso-electric point	2	40	38
Neutral suspension after determining iso-electric point	1	40	39
Purified virus suspension	0	34	46

THE RELATIVE SIZE OF LARYNGOTRACHEITIS AND CORYZA VIRUS PARTICLES

Next, a comparative study of the relative size of laryngotracheitis and coryza viruses was made by filtering each through a graded series of filters following the technique of Allisbaugh and Hyde (1935) and using the formula of Cox and Hyde (1932) for estimating the pore size. It was found that the coryza virus was completely retained by the 4 per cent filter and the laryngotracheitis virus failed to pass through the 5 per cent membrane. These filtration experiments have been repeated ten times for each series of filters, and the results appear to be quite uniform. However, it may not be wise to conclude from these results that the laryngotracheitis virus particle is larger than the coryza virus particle, in view of the greater virulence of the former. It may

be that both viruses passed through the 4 per cent filters in small quantities, and that laryngotracheitis being the more virulent was manifested in the inoculated chickens while the coryza virus had no effect.

Within certain limits it was possible to check the results of the ultrafiltrations by filtering colloidal particles of known dimensions through a duplicate series of collodion membranes. A small unnamed bacterium isolated from the tracheal exudate of a chicken in 1933, and a 0.1 per cent solution of methylene blue, Allisbaugh and Hyde (1935), were selected for these tests. The bacterium was chosen because it represented a small form of

TABLE 3

The comparative ultrafiltration of laryngotracheitis virus, coryza virus, chicken bacterium and methylene blue

COLLODION MEMBRANES	PORE SIZE	LARYNGOTRACHEITIS VIRUS	CORYZA VIRUS	CHICKEN BACTERIUM	METHYLENE BLUE
per cent	*microns*				
1	0.584	+	+	+	+
2	0.231	+	+	0	+
3	0.135	+	+	0	+
4	0.082	+	0	0	+
5	0.045	0	0	0	+
6	0.023	0	0	0	+
7	0.019	0	0	0	+
8	0.013	0	0	0	0
9	0.009	0	0	0	0
10	0.005	0	0	0	0

bacterial life, 0.5μ by 0.1μ, and its presence in the ultrafiltrates could be easily determined by plating in chicken-infusion agar. The methylene blue, 0.03μ to 0.01μ, was selected because it possesses a smaller colloidal particle, and furthermore it aided in detecting bubbles that may have been on the collodion membranes. Its presence in the ultrafiltrates was indicated by its color.

The formula derived by Cox and Hyde (1932) for estimating the pore size of the capillaries in collodion membranes was used for calculating the size of the pores in the graded series of filters directly as the suspensions of laryngotracheitis virus, coryza virus,

chicken bacterium and methylene blue were filtered. The results of these ultrafiltrations are as shown in table 3.

According to the results reported in table 3, the virus particles of laryngotracheitis are less than 0.082μ in diameter, the coryza particles less than 0.135μ, the chicken bacterium less than 0.584μ, and some of the methylene blue colloidal particles less than 0.020μ in diameter. In general these findings are in line with the investigations of Elford (1931), Bauer and Hughes (1934), who state that the particulate size of filterable viruses includes the range of laryngotracheitis and coryza virus particles as estimated in this experiment.

SUMMARY

An improved technique for the ultra-filtration of laryngotracheitis and coryza viruses is described, in which the respective tracheal and nasal exudates are triturated in distilled water with powdered Pyrex glass until the mass is worked into a smooth emulsion. Then, 0.04 molar solution of citric acid is added, drop by drop, until the iso-electric point is reached, and the cellular débris flocculates and begins to settle to the bottom of the tube, leaving an almost clear supernatant suspension of the virus above. The settling of the flocculant material may be hastened by centrifugalization. The supernatant fluid is either poured or pipetted off and neutralized with a 1/25 molar solution of sodium carbonate, using brom thymol blue as an indicator.

The neutralized virus suspension is filtered through a graded series of collodion membranes and the size of its particles estimated. This technique shows some improvements over the methods that are commonly employed in ultrafiltration studies and should find extensive use in bacteriological laboratories for the study of filterable viruses in general.

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STUDIES ON THE EFFECT OF SYNTHETIC SURFACE-ACTIVE MATERIALS ON BACTERIAL GROWTH

I. THE EFFECT OF SODIUM DISECONDARY BUTYL NAPHTHALENE SULPHONATE ON THE GROWTH OF MYCOBACTERIUM SMEGMATIS

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The object of this work is to extend our knowledge of the effects of surface-active compounds on bacterial growth. Previous researches, cited in the reference-list, have concerned themselves with the alkali metal soaps of oleic, ricinoleic, linoleic and other fatty acids, which until recently have been the best known of the surface-active materials of known constitution; recently, however, there have been synthesized a number of compounds possessing a high degree of surface activity. Among these are the alkylated naphthalene sulphonic acids.

These new surface-active compounds have certain characteristics which are not possessed by the fatty acid soaps. Solutions of the alkylated naphthalene sulfonates are extremely stable, being unaffected by organic acids, mineral acids, and alkalies; unlike the soaps, they retain their activity in the presence of lime and magnesium salts, and in general are more resistant to metal salt formation. These compounds differ from soaps in that they lack detergent properties; they are superior to soaps in their ability to decrease solid-liquid interfacial tension, and as penetrating agents. The alkylated naphthalene sulfonates are surface active compounds which depend on the presence of negatively charged colloidal micelles for their surface activity. Being the salts of strong bases and of strong acids, hydrolysis is negligible. The particular material utilized in these experiments was

the sodium salt of the di-secondary butyl naphthalene sulfonic acid. While the exact molecular configuration of this compound is undetermined, the method of synthesis used in its manufacture yields a definitely reproducible material. As available commercially, the material contains about 85 per cent active compound plus 15 per cent sulfate. In view of the interesting properties of these materials, it was felt that an examination of their effect on bacterial growth would be of interest. Previous reports are meager, the only material available being embodied in the patent literature or in the technical literature furnished by the manufacturers. One technical data sheet states that a certain alkylated naphthalene sulfonate "has the property even in very dilute solutions, of retarding the growth of molds and other micro-organisms."

EXPERIMENTAL

Mycobacterium smegmatis was used as the test organism. It was selected because it is a pellicle former, because it is typical of the acid-fast group of bacteria which are especially difficult to "wet out" due to the high concentration of waxy and fatty materials in the capsule, and because it can be cultivated more easily than the pathogenic members of this group. Work is also being conducted with *mycobacterium tuberculosis*. *M. smegmatis* was cultivated in a 5 per cent glycerol broth medium of pH 7.5. The broth, containing various concentrations of the surface active material, was inoculated with a forty-eight-hour growth from the glycerol broth. On inoculation, the pellicle immediately sank to the bottom in all concentrations up to and including 1:10,000, showing that pellicle formation was impossible in these dilutions. In solutions more dilute than 1:10,000, the pellicle floated and grew at a rapid rate. Transfers into plain glycerol broth were made at the end of twelve hours. The results are summarized in table 1.

Smears were made from all dilutions at the end of twelve, twenty-four, and thirty-six hours, stained with carbol-fuchsin, and examined for possible morphological changes. The following variations from the control smears were noted in dilutions through 1:10,000. A magnification of 1,000 diameters was used.

1. Cytolysis had occurred in a great majority of cases. Numerous organisms with a very rough outline were distinctly visible.

2. The majority of the organisms were unevenly stained showing many light spots throughout the cell.

Smears made from dilutions above 1:10,000 showed a variety of sizes and shapes. The organisms were not only smaller in size, but, as mentioned above, involution forms were quite numerous as shown by the fact that the average size of the organisms in dilutions up to 1:50,000 was 1.3μ by 2.5μ as compared to 0.5μ by 4.5μ in plain glycerol broth. Apparently the interfacial tension was sufficiently reduced to allow the bacteria to approach a more spherical shape, in accordance with surface energy considerations.

The hemolytic power of sodium di-secondary butyl naphthalene sulfonate was determined. All solutions of the surface active material, up to and including 1:20,000, were completely hemolytic. It is interesting to note that the hemolytic power is parallel to the bactericidal power of this compound.

TABLE 1

DILUTIONS OF SURFACE TENSION DEPRESSANT IN GLYCEROL BROTH	12 HOURS	24 HOURS	36 HOURS	INOCULATIONS INTO GLYCEROL BROTH AT END OF 12 HOURS
1:100 to 1:10,000	−	−	−	−
1:20,000 to 1:500,000	+	+	+	+

Synergistic properties have been claimed for alkylated naphthalene sulfonates.[1] In order to acquire further data, the effect of Na di-secondary butyl naphthalene sulfonate on the germicidal power of neutral acriflavine was determined. The test organism was *Staphylococcus aureus*. The F. D. A. method of determining phenol coefficient was used. There was no synergistic action. While this does not demonstrate that alkylated naphthalene sulfonates lack synergistic action, it does show that such an effect is not to be expected in every case. To the extent that a surface-active material effects better physical contact between the organism and the germicidal solution, to that extent the germicidal

[1] Chemical Abstracts, 1933, 27, 374.

action is enhanced. But, the presence of a colloidally dispersed material (the surface-active compound) in the solution of the bactericide may decrease the tendency of the bactericide to "escape" from the solution on the cell wall of the bacterium; the degree to which this effect is exerted is dependent upon the particular surface-active material, the germicidal material and the bacteria involved. In a particular case, these two factors, which act in opposite directions, may neutralize each other, with a consequent lack of synergistic action. In other cases, the resultant of these effects may possibly be more favorable, in which case, synergistic action will be encouraged. This question is being investigated further.

CONCLUSIONS

1. The sodium salt of di-secondary naphthalene sulfonic acid, in dilutions up to and including 1:10,000, inhibits the growth of *Mycobacterium smegmatis*; while in dilutions above 1:10,000 and up to 1:50,000 involution forms are very numerous showing that this surface active material is unfavorable for the growth of this acid fast organism.

2. There was no synergistic action between neutral acriflavine and Na di-secondary naphthalene sulfonic acid.

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A SIMPLE APPARATUS FOR POURING AN EXACT QUANTITY OF AGAR INTO PLATES ASEPTICALLY AND FREE FROM FOAM AND BUBBLES

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In the pouring of agar plates there are three factors which are especially important: The thickness of the agar layer, the presence of foam and bubbles on the surface of the agar, and aseptic technique. When agar is poured directly from a flask the variation in thickness of the agar layer may be considerable. Foam and bubbles often form on the surface and these are troublesome to remove. The exposure of the medium to air is considerable and contamination is not uncommon.

The author has perfected a simple device for pouring agar plates rapidly, with an exact quantity of agar into each plate, free from foam and bubbles, and with a lessened chance for air contamination. Figure 1 shows a detailed drawing of the apparatus. The delivery tube is preferably made of pyrex tubing of about 10 to 12 mm. inside diameter and having a total length of 20 to 22 cm. A constriction is made at one end and the inside of the constriction is rounded to form a valve seat. A ball is formed on the end of a glass rod which is the valve stem. This ball is ground into the valve seat with carborundum or emery powder. When the valve holds water, a certain quantity of water such as 13 to 15 cc. is poured into the tube and the height of the meniscus marked on the tube. A hole is then blown in the side of the delivery tube and a side tube of equal diameter sealed in at a slight angle such that the capacity of the delivery tube from the side tube to the valve is about 13 to 15 cc. A small air inlet tube is also sealed in as illustrated. The metal case is rectangular

and may be made of copper, brass, or aluminum. A convenient size is 6 cm. by 3 cm. by 2 cm. A short, 2 cm. metal tube is soldered in at one end as illustrated. All joints should be air tight. A small hole is drilled in the metal case a short distance from the end and near the top. Through this hole is put a stiff wire which serves as an axle for the pendulum. The ends of this

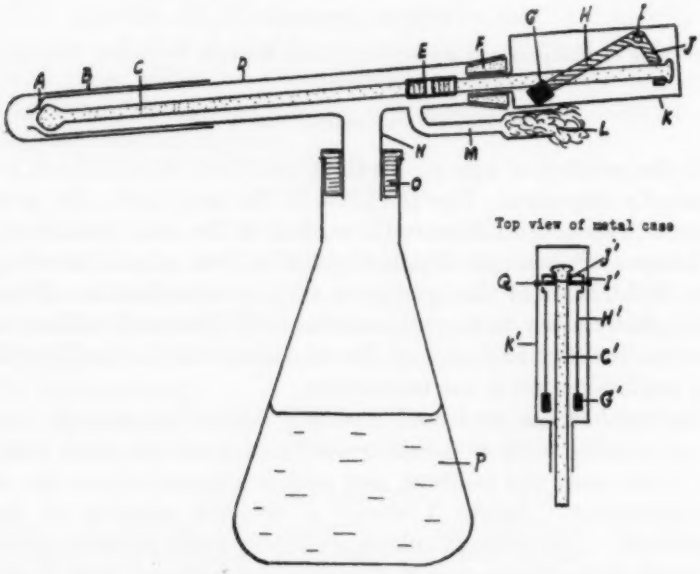

FIG. 1. DRAWING OF APPARATUS FOR POURING AGAR INTO PLATES

A, Valve; *B*, hood; *C*, valve stem; *D*, delivery tube; *E*, rubber connection; *F*, ground glass stopper; *G*, lead weight; *H*, pendulum; *I*, bearing; *J*, elevating arm; *K*, metal case; *L*, cotton plug; *M*, air inlet; *N*, side tube; *O*, rubber stopper; *P*, medium; *Q*, axle.

wire are soldered to the outside of the case. The pendulums and the elevating arm are soldered together at an angle of about 90°. The pendulums may be made out of one long piece of metal furnished with holes and bent into the shape illustrated. The pendulums and elevating arm may also be soldered into a piece of metal tube which fits over the axle. The elevating arm has a hole at the far end through which goes the valve stem. The con-

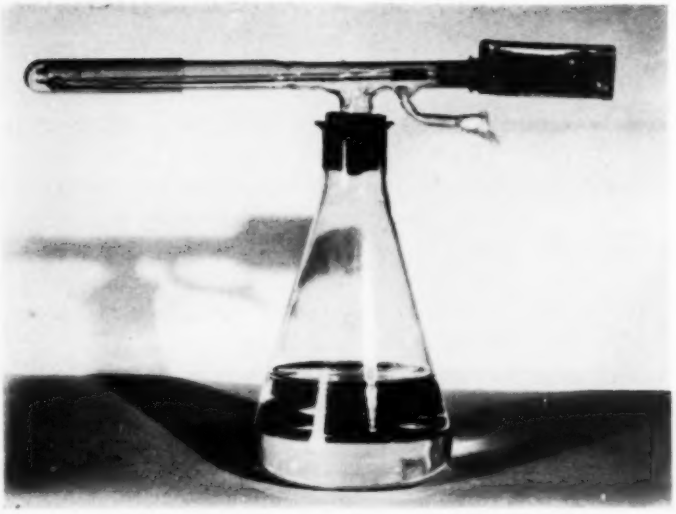

FIG. 2. PHOTOGRAPH OF APPARATUS IN PLACE ON FLASK OF AGAR MEDIUM

FIG. 3. PHOTOGRAPH SHOWING HOW DELIVERY TUBE IS FILLED WITH AGAR

FIG. 4. PHOTOGRAPH SHOWING HOW DELIVERY TUBE EMPTIES AND AGAR RUNS INTO PETRI DISH

UNIV. OF MICH.

nection between the metal case and the delivery tube may be by means of a ground glass stopper, or a threaded metal sleeve may be cemented over the end of the delivery tube and the metal case screwed on. A rubber stopper is not practical for this joint as it is likely to stick after being autoclaved. This joint between the metal case and the delivery tube should be fairly snug, and such that it can readily be taken apart and the delivery tube cleaned with a brush. The agar medium in the flask may be sterilized with the apparatus in place or the two may be sterilized separately. The air inlet tube should be plugged with cotton and the end of the delivery tube protected with a glass hood. Figures 2 and 3 illustrate the technique of pouring plates. When the flask is tilted, the pendulum swings over and closes the valve, and the agar flows into and fills the delivery tube. As the flask is tilted back, the excess agar first drains into the flask and then the valve opens and allows the agar in the delivery tube to flow out. An exact quantity of agar is thus poured into each plate. This method is rapid, no foam or bubbles are carried over with the agar, and the chance for contamination is minimized.

THE METABOLIC ACTIVITY OF VARIOUS COLON-GROUP ORGANISMS AT DIFFERENT PHASES OF THE CULTURE CYCLE[1]

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OBJECTIVES

Earlier contributions from this laboratory (Walker and Winslow, 1932; Walker, Winslow, Huntington and Mooney, 1934) have dealt with the metabolic activity of *Escherichia coli* in a continuously aerated medium; and have brought out the following facts.

The production of carbon dioxide during the first hour of the culture cycle in a peptone-water medium is at the rate of 30 to 40 mgm. $\times 10^{-11}$ per cell per hour. This value rises during the second hour to over 120 mgm. $\times 10^{-11}$ and falls to about 20 mgm. $\times 10^{-11}$ during the fifth hour and to 1 to 3 mgm. $\times 10^{-11}$ after the culture has reached a stable population (twenty-four hours). These variations fit in with the evidence from many observers (see references cited by Walker, Winslow, Huntington and Mooney, 1934) as to the period of physiological youth of the bacterial culture cycle. It seems probable that the high metabolic activity of the early hours—as computed on a cell-per-hour basis—may be largely due to the greater size of the cells at this period (Clark and Ruehl, 1919; Henrici, 1928; Martin, 1932; Bayne-Jones and Adolph, 1932).

The addition of glucose to the peptone-water medium produces no significant effect upon the amount of carbon dioxide produced

[1] This study is based on a thesis presented by G. M. in partial fulfilment of requirements for the Doctorate in Philosophy of Yale University. It was assisted by grants from the Research Fund of the School of Medicine.

in an aerated medium (although the yield of ammonia nitrogen is substantially reduced). The rate of carbon dioxide production in glucose-peptone is actually somewhat less than in plain peptone-water. Under anaerobic conditions, of course, the phenomenon is wholly different. Here, the addition of a fermentable sugar raises the production of carbon dioxide to over 200 mgm. $\times 10^{-11}$ (Walker, Winslow and Mooney, 1934).

The results so far reported have been obtained entirely with *Escherichia coli*. The principles involved are so fundamental that it appeared desirable to repeat the experiments with other species of colon-group bacteria characterized by different fermentative properties.

PROCEDURE

Three species of organisms have, therefore, been employed in the present study. *Escherichia coli* (*Communis* type) was used as a check on the previous work. *Salmonella pullorum* was selected as a type of the organisms which will produce gas from glucose but not from lactose in the ordinary fermentation tube and *Salmonella gallinarum* as an organism which fails to attack lactose and forms acid but no gas from glucose. *Esch. coli* was studied in peptone-water (one per cent) and peptone-glucose medium (1 per cent peptone, 0.5 per cent glucose); the other species were observed in these same media and also in peptone-lactose medium (1 per cent peptone, 0.5 per cent lactose). In working with *Sal. pullorum* in the peptone-glucose medium we found it necessary to add 0.5 per cent NaCl. This organism increased rapidly in numbers in the peptone-water and peptone-lactose media but entirely failed to multiply in the aerated standard peptone-glucose medium even after ten hours. It appeared that the glucose in an aerated medium exerted an inhibitive effect. A check culture in the ordinary laboratory fermentation tube, which contained 0.5 NaCl in addition to glucose and peptone multiplied rapidly; and, with the addition of salt, normally high numbers appeared under aeration. Why glucose should be inhibitive in an aerated culture and why this inhibition should be removed by the presence of NaCl we have not yet discovered.

Our general procedure was the same as that previously described by Walker (1932) and by Walker and Winslow (1932). It involved the cultivation of the organisms in Dreschel bottles kept in a water-bath at 37°C. and continuously aerated with air freed from carbon dioxide and ammonia. Two duplicate cultures and one uninoculated control were kept under simultaneous observation for twenty-four hours. Bacterial counts were made in triplicate by standard plating methods at hourly intervals for the first seven to ten hours and twice more between the twenty-second and twenty-eighth hours. At the same periods a sample of the medium was analyzed for dissolved carbon dioxide and the yield of carbon dioxide carried off in the aeration train was determined by titration. The apparatus and method of analysis have been fully described in the papers cited above.

In the experiments with *Sal. pullorum* and *Sal. gallinarum*, electrometric determinations of pH were made with the quinhydrone electrode.

It will be noted that eight different series of experiments were made, as follows: *Esch. coli* in peptone-water and peptone-glucose medium; *Sal. pullorum* in peptone-water, peptone-glucose and peptone-lactose; *Sal. gallinarum* in peptone-water, peptone-glucose and peptone-lactose. For *Sal. gallinarum* in the peptone-glucose medium seven duplicate experiments were run and, for all other conditions, six duplicate experiments. These duplicate experiments checked reasonably well but naturally showed individual variations. For the purpose of the present discussion we have used the means of the six (or seven) experimental findings for a given organism in a given medium.

GROWTH CURVES OF THE THREE ORGANISMS IN VARIOUS MEDIA

The average results of our eight series of experiments—so far as bacterial multiplication is concerned—are summarized in table 1.

These results are plotted on a logarithmic scale in figure 1.

The curves seem to indicate that our cultures approximately reached their peak after ten hours (except possibly in the case of *Esch. coli* in peptone-water). The second-day counts were about on the level of the final first-day counts except in the

case of *Sal. pullorum* in peptone-water (which was on a lower level) and of *Sal. gallinarum* which was always falling rapidly in numbers at the twenty-four-hour period. It will be noted that results for *Sal. pullorum* in peptone-water are not directly comparable with the others, since the initial population in this series was only about 5 million instead of 10 to 16 million.

The three organisms show rather definite and characteristic differences in their rates of increase. *Esch. coli* has a much shorter lag period (one hour) than the Salmonellas followed by a

TABLE 1

Mean population of bacteria after various time intervals

TIME INTERVAL AFTER INOCULATION	ESCH. COLI*		SAL. PULLORUM*			SAL. GALLINARUM*		
	Peptone	Glucose-peptone	Peptone	Glucose-peptone	Lactose-peptone	Peptone	Glucose-peptone	Lactose-peptone
hours								
0	13.6	15.2	5.3	15.7	9.9	11.3	11.8	13.2
1	13.9	13.3	5.6	11.7	13.3	10.9	12.9	13.3
2	20.8	37.8	5.9	9.9	15.7	11.2	16.4	10.4
3	51.7	154.5	6.9	13.0	18.7	12.2	18.4	10.9
4	168.4	293.1	10.3	16.2	31.5	21.8	32.3	12.1
5	294.5	447.8	19.2	38.9	53.2	68.2	97.7	24.1
6	752.8	465.6	39.1	70.3	93.4	171.1	349.6	90.4
7	1183.3	555.6	67.4	320.2	203.9	278.9	442.9	383.3
8			128.7	603.4	326.7	558.9	636.7	650.0
9			333.1	705.8		626.0	690.4	
10			419.4			758.3	736.2	
23	1333.3	405.6	95.3	356.7	308.3	283.3	551.0	81.3
25	1115.0	444.5	104.7	331.7	249.5	50.8	283.8	26.9

* The figures represent millions per cubic centimeter.

sharp rise during the logarithmic phases. Its ultimate level is very high in peptone-water. *Sal. pullorum* shows a longer lag period (two to three hours) and a less rapid logarithmic increase than either of the other species. *Sal. gallinarum* shows a long lag period (three to four hours) a steep logarithmic rise (about the same as *Esch. coli*) and a sharp fall on the second day. It will be noted that after seven hours *Esch. coli* had a population of nearly 1200 million per cubic centimeter in peptone-water and 550 million in glucose-peptone. The other cultures showed at

this time populations between 200 million and 450 million (except for *Sal. pullorum* in peptone-water where the initial inoculum was so low).

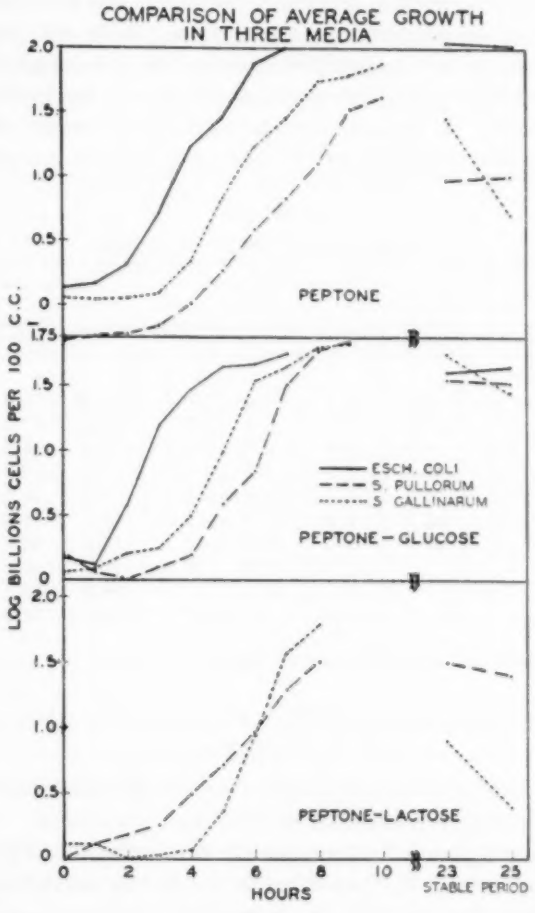

FIG. 1

The effect of the different media upon a given organism was much less marked. With *Esch. coli*, the glucose-peptone medium showed a sharper but briefer logarithmic rise with an ultimately

lower population. The lower final level of population may very probably be due to hydrogen ion concentration, as in the studies of Cohen and Clark (1919). With *Sal. pullorum* the logarithmic rise was again steeper in glucose-peptone but was not abbreviated and yielded the highest final population. With *Sal. gallinarum* the average curve was again steeper in the glucose-peptone medium, showing a greatly increased population from the fifth to the eighth hours. It appears certain that the presence of glucose stimulates the sharpness of the later logarithmic growth phase.

TABLE 2

Cumulative carbon dioxide production

TIME INTERVAL AFTER INOCULATION	ESCH. COLI*		SAL. PULLORUM*			SAL. GALLINARUM*		
	Peptone	Glucose-peptone	Peptone	Glucose-peptone	Lactose-peptone	Peptone	Glucose-peptone	Lactose-peptone
hours								
1	0.8	0.6	0.4	0.1	0.5	0.04	0.4	0.3
2	2.6	2.5	0.9	0.4	0.9	0.4	1.3	1.0
3	5.5	8.8	1.6	0.8	2.3	1.4	2.8	2.8
4	13.1	20.6	2.5	2.2	4.1	3.7	6.3	5.1
5	25.8	30.2	3.8	5.2	6.2	6.5	12.5	9.8
6	39.4	39.4	5.7	10.2	9.8	10.7	21.6	15.3
7	51.8	47.8	8.6	19.6	15.4	15.6	32.8	21.1
8			13.2	31.0	21.2	21.7	42.6	27.2
9			18.4	42.4		28.3	49.4	
10			25.5			33.2	54.9	

* The figures represent milligrams per 100 cc.

CARBON DIOXIDE PRODUCTION OF THE THREE ORGANISMS IN VARIOUS MEDIA

The average cumulative yields of carbon dioxide in the various experiments are summarized in table 2.

The results parallel rather closely the growth curves as previously analyzed. Carbon dioxide yields are markedly higher for *Esch. coli* than for the other organisms, from the second hour on. They are higher in glucose-peptone than in peptone-water from the third to the fifth hour for *Esch. coli*. They are higher in glucose-peptone than in either of the other media from the seventh hour on for *Sal. pullorum* and from the sixth hour on for

Sal. gallinarum. (Results for *Sal. pullorum* in peptone-water are not comparable on account of the initial low inoculum.) All these results are what would be expected from the counts in table 1.

In table 3 and in figure 2 these results are re-computed on a basis of yield per cell per hour. The method of computation is the same as that used by Walker and Winslow (1932).

TABLE 3

Mean rate of carbon dioxide production during various time intervals

TIME INTERVAL	ESCH. COLI*		SAL. PULLORUM*			SAL. GALLINARUM*		
	Peptone	Glucose-peptone	Peptone	Glucose-peptone	Lactose-peptone	Peptone	Glucose-peptone	Lactose-peptone
hours								
0- 1	53	44	78	6	39	4	35	23
1- 2	108	79	80	30	30	29	57	53
2- 3	86	77	121	33	84	87	86	166
3- 4	77	54	105	99	73	144	145	205
4- 5	56	26	91	114	51	67	104	269
5- 6	28	20	67	96	50	38	46	111
6- 7	13	17	56	57	40	23	29	29
7- 8			48	26	22	14	18	12
8- 9			24	17		11	10	
9-10			19			7	8	
23-25	2	4	6	8	3	5	3	6

* The figures represent milligrams $\times 10^{-11}$ per cell per hour.

The results presented in table 3 and figure 2 may be briefly summarized as follows:

Esch. coli shows a rate of carbon dioxide production of about 50 $\times 10^{-11}$ mgm. during the first hour. The rate rises to between 80 and 110 during the second hour and then falls steadily to about 15 during the seventh hour and to less than 5 on the second day. These results closely check those reported in our earlier studies. Again, as in these earlier studies, the rate of carbon dioxide production is lower in glucose-peptone than in peptone-water in spite of the fact that the glucose is actively attacked, as shown by the change in hydrogen-ion concentration. At the end of the seventh hour the pH was 7.8 in peptone-water and 5.4 in peptone-glucose. Yet the average rate of carbon dioxide yield for the

first seven hours was 60 in peptone-water and 45 in peptone-glucose (under anaerobic conditions the phenomenon is quite different, the rate of carbon dioxide production rising to 211 as shown by Walker, Winslow and Mooney, 1934). The fact that the presence of glucose accelerated the rate of multiplication during the logarithmic phase seems at first sight inconsistent with the lower rate of carbon dioxide yield. It may, however, be that the more rapid rise of numbers in glucose-peptone is

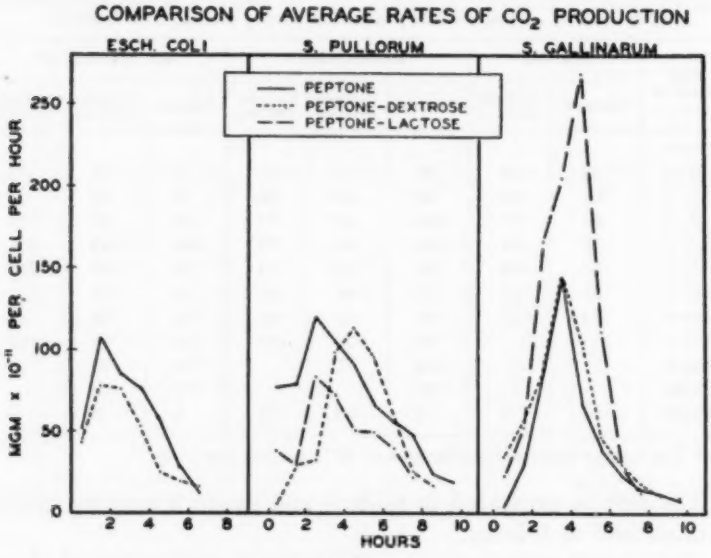

FIG. 2

associated with smaller cell-size which would account for the lowered yield of carbon dioxide per cell.

Sal. pullorum exhibits in peptone-water much the same phenomena exhibited by *Esch. coli* with the single exception that the rate of carbon dioxide formation during the first hour was somewhat high. The lactose-peptone medium showed lower rates throughout than did the peptone-water medium—just as with *Esch. coli* the glucose-peptone rate was lower than the peptone-water rate. In this case, however, the sugar was not at-

tacked, the pH in both peptone-water and lactose-peptone remaining at about 7.3. The curve in glucose-peptone is, on the other hand, a wholly different one (figure 2). The rise in rate of production of carbon dioxide is much slower, with a peak in the fifth instead of the third hour; and its subsequent fall is more rapid than that of the other curves for *Sal. pullorum* and *Esch. coli*. It will be recalled that this was the medium which proved inhibitory to *Sal. pullorum* and in which this organism would not multiply at all without the addition of NaCl. It is evident that, while the presence of salt permitted a normal growth curve, the metabolic activity of the organism was still inhibited.

Sal. gallinarum in all three media shows a curve which is characteristic and different from the curve for the other organisms. *Esch. coli* and *Sal. pullorum* (except in glucose peptone) show their peak-rate of carbon dioxide yield in the second or third hour, while with *Sal. gallinarum* the peak occurs in the fourth or fifth hour. The peak itself is high (exceedingly high in lactose-peptone); the drop after the peak is very sharp. This sharp drop is conditioned by the high and late peak, which is really the characteristic feature of the organism.

It will be noted by reference to tables 1 and 2 that the enormous peak in carbon dioxide yield per cell during the fourth and fifth hours with *Sal. gallinarum* in peptone-lactose is due to a normal total carbon dioxide yield and a very low cell count. In the first experiments with this organism in this medium, on noting a normal turbidity in the flasks and making dilutions accordingly, we were much puzzled by obtaining only sterile plates in many instances. Microscopic dark-field examinations made by Dr. H. H. Walker, as a part of a concurrent investigation showed that at this particular part of the life cycle the organism exhibited a considerable proportion of rather long chains of streptobacilli. This was no doubt a partial explanation of the low plate counts and the high yield of carbon dioxide per cell per hour.

It is of interest to note that the three organisms show much the same characteristics as regards carbon dioxide yield per cell which they exhibit in regard to multiplication. *Esch. coli* shows a quicker and sharper logarithmic rise in numbers than the other

organisms; it also exhibits an earlier and more rapid rise in carbon dioxide yield per cell. *Sal. pullorum* has a longer lag in growth and also in carbon dioxide yield per cell. *Sal. gallinarum* shows a sharp rise and a subsequent very sharp fall. Evidently both morphological and physiological factors are related to somewhat deep-seated characters of the organisms in question.

GENERAL DISCUSSION

The first point of interest brought out in this study is a confirmation of the earlier findings of Walker and Winslow (1932) and Walker, Winslow, Huntington and Mooney (1934) that in an aerated medium the addition of a fermentable sugar tends to decrease rather than to increase the yield of carbon dioxide per cell per hour with *Esch. coli*. In the present experiments the carbohydrate was actively attacked by *Esch. coli*, by *Sal. pullorum* and by *Sal. gallinarum* in peptone-glucose, as evidenced by a pH of 4.5 to 4.7 after twenty-four hours. The other five combinations of organisms and media showed a pH of 7.9 to 8.2 at this period. Yet the average results for the first seven hours for carbon dioxide yield per cell (in mgm. $\times 10^{-11}$) were as follows.

	Peptone-water	*Peptone-glucose*	*Peptone-lactose*
Esch. coli	60	45	
Sal. pullorum	85	62	52
Sal. gallinarum	56	72	122

The lowest rates (*Esch. coli* in both media, *Sal. pullorum* in peptone-lactose and *Sal. gallinarum* in peptone-water) correspond with rapid multiplication; the highest yields (*Sal. pullorum* in peptone-water and *Sal. gallinarum* in lactose-peptone) correspond with slow multiplication. The cumulative yield of carbon dioxide on the other hand is more consistent than the increase in numbers, which suggests the probability that variations in the size of cell aggregates may largely account for the variations observed. We suspect this to be particularly the fact with *Sal. gallinarum* in lactose-peptone. In any case there is clear evidence that decomposition of sugar does not increase carbon dioxide yield per cell. With *Esch. coli* the yield per cell is lower in the presence of glucose than in peptone-water; and with the

other two organisms the rate in glucose media is intermediate between the rates in peptone-water and in lactose-peptone, where no fermentation occurs.

Comparison of the different organisms is made a little difficult on account of the fact that the peak rate of carbon dioxide yield comes at different periods. For all organisms, we find a maximum yield of carbon dioxide between 79 and 145 mgm. $\times 10^{-11}$ per cell per hour (see table 3) and a minimum (on the second day) of 2 to 8 mgm. Where the peak comes during the second hour (*Esch. coli*) the first-hour rate is high (44 to 53 mgm.); where the peak comes in the third hour (*Sal. pullorum* in peptone and peptone-lactose) it is also high (39 to 78 mgm.); where the peak does not occur until the fourth hour (*Sal. gallinarum* in peptone and peptone-lactose), the first-hour rate is lower (4 to 35 mgm.); and where the peak does not come till the fifth hour (*Sal. pullorum* in peptone-glucose) the first-hour rate is as low as in the second-day stable phase. Data for *Sal. gallinarum* in peptone-lactose may be omitted from consideration on account of the complicating factor of chain formation.

We are inclined to believe that the curve for *Sal. pullorum* in peptone-glucose is the most significant of the four types of reaction presented in table 3, since it gives a sort of slow-motion picture of phenomena which are obscured by the more rapid changes in the other media. It seems reasonable to believe that in all cases there is a certain period after inoculation during which the rate of carbon dioxide yield is at the level of the stable phase (2 to 8 mgm. $\times 10^{-11}$). This period lasts through the whole of the first hour with *Sal. pullorum* in glucose-peptone and with *Sal. gallinarum* in peptone-water and so we obtain data similar to the twenty-four-hour figures. In the other instances this relatively inert phase of metabolic activity presumably lasted for only a part of the first hour and was therefore undetected. There seems to us no reason to assume any fundamental difference in metabolic activity between the various organisms in the various media studied other than those differences due to rate of multiplication (which affects the period at which a certain metabolic rate may occur) and to size of cells.

As to the possible influence of cell-size, the results of Bayne-

Jones and Adolph (1932) show that the range of cell volume for *Esch. coli* at various phases of the culture cycle is not over ten to one. On the other hand, the carbon dioxide yield per cell varies from 2 to 108 mgm. $\times 10^{-11}$, a fifty-fold variation. Clearly the differences in carbon dioxide can only in part be accounted for by change in cell size. We must conclude that the phase of physiological youth in the culture cycle is characterized, not only by increase in cell size, but also by an even greater increase in metabolic activity per unit mass of living protoplasm.

In general the work of Henrici, Martin, Bayne-Jones and Adolph as summarized by Walker, Winslow, Huntington and Mooney (1934) indicates that the size of the colon bacillus at

TABLE 4

Production of carbon dioxide by various types of living organisms

	GRAMS PER HOUR PER KILOGRAM OF BODY WEIGHT		GRAMS PER HOUR PER KILOGRAM OF BODY WEIGHT
Cestus veneris (coelenterate)	0.004	Melolontha (insect)	1.1–1.7
Mytilus (mollusc)	0.017	Dog	1.3
Lumbricus (earthworm)	0.108	Hen	1.3
Collozoum (protozoan)	0.111	Cat	1.4
Cancer (crab)	0.17	Mouse	8.4
Man	0.33	Sparrow	10.5
Ox	0.39–0.49	Bacteria	40.0–330.0

various periods of the life cycle varies from about 1.75 $\times$.65 micra to 4.5 $\times$1.0 micra which would give corresponding bacterial volumes of 0.5 and 3.25 cubic micra respectively.[2] If we combine our lowest rate of carbon dioxide production (2 mgm. $\times 10^{-11}$ per cell per hour) with the smallest of these cell sizes we obtain a yield of 40 grams of carbon dioxide per kilogram of bacterial substance; while our highest rate for *Esch. coli* (108 mgm. $\times 10^{-11}$ per cell per hour), combined with the largest size of cell, gives a yield of 330 grams of carbon dioxide per hour per kilogram of bacterial substance. In table 4 we have com-

[2] Studies now in progress in this laboratory by Miss Evelyn Huntington confirm these general relationships for our own strain of colon bacilli.

pared these results with those for certain other forms of life as cited by Macleod (1922) and by Rogers (1927). It is obvious that the bacteria, even in the second-day stable phase of the growth cycle are metabolically far more active than any of the animal forms recorded by the authors in question.

CONCLUSIONS

The studies here reported deal with the production of carbon dioxide by *Escherichia coli*, *Salmonella pullorum* and *Salmonella gallinarum* in continuously aerated media at various periods of the culture cycle. They appear to warrant the following conclusions.

1. The presence of a fermentable sugar (glucose) in an aerated medium stimulates the multiplication of *Escherichia coli* and *Salmonella gallinarum* but tends to inhibit the development of *Salmonella pullorum*. This inhibitive effect can be removed by the addition of NaCl. In no case is the presence of glucose accompanied by any significant increase in the rate of production of carbon dioxide per cell per hour.

2. The production of carbon dioxide per cell per hour at a corresponding period of the culture cycle seems to be essentially independent of the species of organism studied and of the medium used. It would appear therefore to represent a fundamental metabolic activity which is of the same order of magnitude in the three organisms.

3. At various phases of the culture cycle, however, the rate of metabolic activity varies very widely. On a cell per hour basis it may rise to a figure as high as 79 to 145 mgm. $\times 10^{-11}$ at the beginning of the phase of logarithmic increase and thereafter falls to 2 to 8 mgm. $\times 10^{-11}$ after twenty-four hours when the culture has reached a stage of stable population. Minor variations in the rapidity with which metabolic activity rises to, and falls from, its peak, with different organisms, and in different media are rather closely paralleled by the differences between the growth rates of the corresponding organisms in the corresponding media.

4. High rates of carbon dioxide production per cell per hour

in the early phases of the growth cycle can in part, but only in part, be explained by increased cell-size at the same period. Comparison with the work of earlier observers on cell size suggests that when the volume of bacterial substance per cell increases tenfold the yield of carbon dioxide increases fifty-fold. It is therefore evident that the period of physiological youth in a bacterial culture is characterized not only by larger cells but by cells which are much more active metabolically per unit of living matter concerned.

5. Computations of carbon dioxide production per unit body weight show that the bacteria here studied are, metabolically, enormously more active than other living organisms previously observed. Even in the stable second-day phase of the culture cycle they are four hundred times as active as the protozoan, *Collozoum*, one hundred times as active as man and four times as active as the sparrow, which shows the highest metabolic activity which we have found on record. In the period of physiological growth these ratios may be multiplied tenfold.

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PROCEEDINGS OF THE LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW YORK CITY BRANCH

THIRD MEETING, COLLEGE OF THE CITY OF NEW YORK, FEBRUARY 19, 1935

THE RÔLE OF BACTERIA AND OTHER MICROORGANISMS IN THE DECOMPOSITION OF CELLULOSE IN NATURE. *Selman A. Waksman*, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Microorganisms capable of decomposing cellulose are widely distributed in nature. They are active under a variety of conditions, such as soils and peat bogs, manure composts and sewage disposal plants, lake, river and ocean waters, in the rotting of vegetables in storage and in the digestive tract of herbivorous animals. Biologically, they belong to a number of groups, comprising largely fungi, actinomyces and bacteria. Among the fungi, they are found among the *Ascomycetes*, *Hyphomycetes* and *Basidiomycetes*. The cellulose-decomposing bacteria comprise a number of aerobic, anaerobic and thermophilic types, which vary considerably both in their morphological and physiological characteristics.

The mechanism of cellulose decomposition varies widely. It is assumed that the cellulose is first hydrolyzed to cellobiose and the latter to glucose. However, there are only few experiments on record where the production of glucose by cellulose-decomposing bacteria has actually been established. Many of the bacteria which decompose the cellulose synthesize considerable cell substance of a slimy nature and consisting largely of certain hemicelluloses belonging to the polyuronide type. The anaerobic bacteria decomposing cellulose produce a variety of organic acids and alcohols, which can be utilized for industrial purposes.

THE FUNGICIDAL ACTION OF SULPHUR. *Frank Wilcoxon and S. E. A. McCallan*. Boyce Thompson Institute, Yonkers.

Two theories have been advanced in recent years to explain the action of sulphur as a fungicide: (a) that traces of pentathionic acid in the sulphur constitute the active fungicidal agent; (b) that H_2S formed from the sulphur is the active agent. The authors prepared pure potassium and barium pentathionates, and from these obtained solutions of pentathionic acid. The toxicity of the acid was insufficient to account for the toxic action of sulphur to fungous spores. H_2S was much more toxic. It was found that sulphur dust in contact with leaves or fungous spores gives rise to measurable quantities of H_2S. This reduction was followed quantitatively, and the influence of temperature on the reaction was studied. The optimum temperature is about 33°C. Above 55° no H_2S is formed. The amount of H_2S formed varies with the species of spores

used. The order of susceptibility of eight species of fungi to sulphur, and to H_2S is the same. The production of H_2S takes place on or in the spores, and the reaction may take place across an air space of 2 to 3 mm. It is suggested that glutathione is concerned in the reduction of the sulphur, and that the ability to reduce sulphur is a function of the living spores. Selenium and tellurium are much less toxic than sulphur, although their hydrides are highly toxic.

Acid Production and Respiratory Catalysts of Escherichia Acidilactici. *Kenneth C. Blanchard*, Washington Square College, New York University.

In the fermentation of glucose by *Es. acidi-lactici*, the quantity of lactic acid formed per mol of glucose fermented has been found to be dependent upon both the composition of the medium and the availability of oxygen to the organism. For a given medium the yield of this acid in aerobic cultures is approximately twice that obtained in anaerobic cultures. That this curious behavior is dependent upon the accessibility of oxygen is shown by the fact that in the presence of a suitable concentration of cyanide the aerobic yield of lactic acid obtainable from cultures in nutrient broth may be depressed to essentially the anaerobic level.

Carbon monoxide acts quite differently from cyanide. At partial pressures below 0.9 atmosphere this gas is without effect upon the aerobic yield of lactic acid, while at higher partial pressures the aerobic production of lactic acid exceeds that characteristic of aerobic cultures.

From these results it is concluded that *Es. acidi-lactici* contains at least two oxygen-activating mechanisms which have different functions in the metabolism of the cell.

While this organism is capable of rapid growth during anaerobiosis, no growth occurs in an atmosphere of pure carbon monoxide. From this it appears that the carbon-monoxide-sensitive catalysts of the cell are concerned with the synthesis of cellular constituents as well as with oxygen activation.

The Rôle of Glutathione in the Metabolism of Yeast. *Samuel Machlis and Kenneth C. Blanchard*, Washington Square College, New York University.

Six strains of yeast were examined with respect to the comparative magnitudes of their respiration, aerobic fermentation, and content of reduced and oxidized glutathione, with a view towards elucidating the rôle of this substance in carbohydrate metabolism. In air the ratio of the number of mols of glucose oxidized to the number fermented varied in the different yeast strains from $\frac{1}{2}$ to $\frac{1}{10}$ and the total glutathione content of the individual strains was found to vary in a parallel manner. In all strains of yeast examined, the ratio of oxidized to reduced glutathione was found to be sensibly the same.

Narcotics such as ethyl urethane, exposure to hydrogen peroxide, variations in pH, and enforced anaerobiosis, were found to be without effect upon the ratio of the two forms of glutathione. Exposure to iodine, iodoacetic acid and phenyl mercuric chloride, all of which react with glutathione and thereby diminish the total effective intercellular concentration of this substance, proved to be without effect upon the above ratio.

From these experiments it appears that in the yeast cell the glycolytic and oxidative degradation of glucose

are interrelated processes, both of which are dependent upon the total glutathione content.

THE INDUSTRIAL PRODUCTION AND USES OF YEAST. *Charles N. Frey*, The Fleischman Laboratories.

FOURTH MEETING, TEACHERS' COLLEGE, COLUMBIA UNIVERSITY, MAY 14, 1935

RESPONSE OF TUBERCULOUS ANIMALS TO TOXINS. *Jules Freund*, Cornell University Medical College.

Products of *Escherichia coli*, typhoid bacilli or meningococci are only slightly toxic for normal rabbits or guinea pigs since several cubic centimeters of these toxins are necessary to produce toxic symptoms or death in normal animals. In contrast to normal animals, tuberculous rabbits or guinea pigs die from a few tenths of a cubic centimeter of these toxins with characteristic symptoms. Death follows the injection of toxins within a few minutes to a few hours, with intense congestion of blood vessels, hemorrhages into organs containing tubercles and exudation into the serous cavities. In guinea pigs convulsions, labored breathing, i.e., anaphylactoid symptoms, are often seen.

This phenomenon is non-specific. Not only tuberculous animals but guinea pigs infected with cholera vibrios and some other bacteria succumb to the toxic bacterial products mentioned. This heightened susceptibility is different from anaphylaxis. Guinea pigs injected with dead tubercle bacilli tolerate large amounts of toxins.

These observations show in experimental animals that a focus of infection makes the host susceptible in a peculiar manner to the action on intercurrent diseases, of various non-related injurious agents the focus of infection acting as *locus minoris resistantiae*.

A STUDY OF THE ORGANISMS RECOVERED FROM FILTRATES OF HEMOLYTIC STREPTOCOCCUS CULTURES. *Edith E. Nicholls*, New York Hospital.

Ninety cultures from filtrates of seven biologically identical strains of hemolytic streptococcus were made and the ninety filtrates obtained were studied by means of 627 series of washings or 4655 total washings. A streptococcus was recovered from 28 or 31.1 per cent of the filtrates and 4.4 per cent of the series of washings. A diphtheroid-like bacillus appeared in 23.4 per cent of the series and other organisms such as *Staphylococcus albus*, *Bacillus subtilis*, and *Bacillus proteus* in 16.4 per cent of the series. Neither the type of medium used nor the amount, had any influence on the percentage of streptococci recovered from subsequent filtrates. Filtrates from cultures incubated for twenty-four hours and allowed to evaporate in the ice-box yielded a markedly higher percentage of streptococci than those left in the room or incubator.

All of the streptococci were of the viridans type when they first appeared. Six on transfer became hemolytic, 20 remained the same after numerous transfers into various mediums and 2 later showed no change in the blood agar. The hemolytic strains gave the same sugar reactions as the original strains while the viridans strains showed great variation.

Nine of the tubes were passed through an L5 filter and twenty-five serial washings were carried out. No streptococci were recovered. One filtrate was found to contain a diphtheroid-like bacillus when subcultured into blood broth. A *Staphylococcus albus* appeared on four plates. In 20 in-

stances 0.5 cc. of sterile broth was washed over a plate and subcultured ten times. A diphtheroid-like organism appeared only once and the other plates remained sterile.

From this study it would seem that the streptococci recovered represent either very minute streptococci or small modified forms that pass through the filter and on culture develop into the original form of streptococcus or one with modified cultural characteristics. Because of the fact that the diphtheroid-like bacilli were recovered from the test filtrates and also from the controls, although in a lower percentage in the latter case, it would seem reasonable to assume that these were contaminations.

THE INCREASE IN BACTERICIDAL VALUE OF VAPORS FROM IRRADIATED ESSENTIAL OILS. *W. Reiner-Deutsch and N. Molnar.*

Irradiated essential oils were studied in respect to their bactericidal properties. Irradiation of these oils in alcohol for fifteen minutes at a distance of twenty-five centimeters, using a Burdick air-cooled lamp, increased the bactericidal value to a point at which the vapors from 0.2 cc. killed *Staphylococcus aureus* cultures in three hours. Photochemical studies of the irradiated oils showed a marked radiation which decreased in time. After five months the bactericidal power also lessens slightly. Further studies are being conducted on the possible bactericidal agent and on the cause of decrease in this effect.

THE IMMUNOLOGICAL RESPONSE FOLLOWING THE INGESTION OF FOOD PROTEINS BY NORMAL INFANTS. *Vernon W. Lippard*, New York Hospital.

Four hundred and sixty complement fixation tests for the presence of antigenic lactalbumin and the antibodies for cow's milk proteins and lactalbumin were performed on 229 normal infants, children and adults. A number of infants were examined repeatedly over a period of 13 months.

As a result of this investigation, it was found that following the initial ingestion of cow's milk by normal infants, there is absorption of a small amount of cow's milk protein in antigenic form. The presence of this circulating antigen leads to the production of specific antibodies which are demonstrable in the blood stream over a period of several months. Despite the continued ingestion of cow's milk, both cow's milk proteins and their specific antibodies subsequently disappear from the circulation.

The results obtained for cow's milk were confirmed in a study of the ingestion of egg white.

DIFFERENTIAL STAINING OF THROAT EPITHELIAL CELLS AND INVADING ORGANISMS. *Jean Broadhurst, Rosamond Liming, and M. Estelle Maclean*, Teachers' College, Columbia University.

These observations are based on routine examinations of human throats (normal individuals with the usual percentage of ambulatory cases of colds, sore throats and "chronic sinuses") and laboratory animals (dogs, cats, guinea pigs, rabbits and monkeys) inoculated from human throats containing the cytoplasmic bodies reported at the May, 1934, meeting of this Chapter of the S. A. B.

Among the stains demonstrated are:

(1) Nigrosin (one-half aqueous concentration, 7 to 10 minutes). This blots out with remarkable uniformity all bacteria and fungi including yeasts, and food particles.

(2) Acetic acid followed by nigrosin. To avoid possible confusion with mito-

chondria the smears are treated with 1 to 5 per cent acetic for 1 to 24 hours.

(3) William's stain and Seller's stain for Negri bodies. With each of these stains differentiation of the cytoplasmic bodies was best obtained by following the stain with a quick dip in alcohol (95 per cent).

(4) Differential stains for animal tissues

(a) Since these inclusion bodies stain vividly with all bacterial nuclear stains, we tried Nucplascoll as a differential stain. The bodies are a green, sometimes stippled, especially in the center, and definitely capsulated, the cytoplasm of the epithelial cells is pale gray to a pale green-gray and the nuclei are pinkish. Nigrosin followed by Nucplascoll gives very distinct black for the cytoplasmic bodies; bacteria and fungi range from pale gray to reddish purple.

(b) Better contrast was obtained by the following procedure: (1) Salt solution; (2) Nigrosin; (3) Feulgen's stain (for nuclei); and (4) Dilute magenta. With this serial treatment the bodies are black, the nuclei of the epithelial cells pale blue and the cytoplasm pink.

CENTRAL NEW YORK STATE AND CENTRAL PENNSYLVANIA BRANCHES

COMBINED SPRING MEETING, CORNELL UNIVERSITY, ITHACA, NEW YORK, MAY 25, 1935

BETA HEMOLYTIC STREPTOCOCCI ASSOCIATED WITH SEPTIC SORE THROAT EPIDEMICS. *G. J. Hucker*, New York State Agricultural Experiment Station, Geneva, New York.

A COURSE IN GENERAL BACTERIOLOGY FOR THE COLLEGE STUDENT. *C. A. Darling*, Allegheny College, Meadville, Pennsylvania.

COMPARATIVE PRODUCTIVITY TESTS OF PRESUMPTIVE TEST MEDIA WITH PURE CULTURES OF THE COLI-AEROGENES GROUP. *Michael A. Farrell*, The Pennsylvania State College, State College, Pennsylvania.

Lactose broth, buffered lactose broth, brilliant-green lactose bile 2 per cent, fuchsin lactose broth, methylene-blue brom-cresol purple broth, crystal-violet lactose broth, formate-ricinoleate broth, peptone lactose bile broth and trypaflavine broth were compared by determining their productivity using the Butterfield method with 15 recently isolated pure cultures of the coli-aerogenes group.

The results indicate that standard lactose broth was the most productive for these cultures. Giving this medium a value of 100 and calculating the sensitivity of the other media in terms of lactose broth positives, the following values were determined: fuchsin lactose broth 98.5 per cent; brilliant-green lactose bile 94.2 per cent; and buffered lactose broth 92.0 per cent. The remaining 5 media showed less than 85.0 per cent the productivity of standard lactose.

AGAR DIGESTING BACTERIA. *Harry E. Goresline*, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

THE FORMATION OF FATTY ACIDS BY ASPERGILLUS NIGER. *C. F. Schmidt, Jr.*, University of Rochester, Rochester, New York.

A study has been made of the formation of fatty acids by *Aspergillus niger* under different environmental conditions using methods previously developed (Jour. Biol. Chem., 1935, **110**,

511-520) by which it is possible to separate the processes of growth and the processes of fatty acid formation. It has been found that sodium fluoride in 0.001 M concentration stimulates the formation of fatty acids from glucose and that 0.0035 M completely inhibits the process. Fatty acids are formed from pyruvic acid and this process is not inhibited by 0.0035 M sodium fluoride. These results are considered significant in relation to the mechanism of fatty acid formation.

The relative amounts of fatty acids formed from glucose, sucrose, levulose, mannitol and glycerol have been determined. Comparisons between glucose and sucrose indicate that concentration may affect the relative value of the sugar for fatty acid formation. A comparison of fatty acid formation from glucose in concentrations of from 5 to 60 per cent indicates the existence of an optimum concentration in the region of from 20 to 40 per cent and higher concentrations have been found less effective in causing fatty acid formation.

A Critical Consideration of Bacteriophage Therapy in Staphylococcus Infections. *Merl G. Colvin*, Williamsport, Pennsylvania.

In view of the inconclusive evidence on the relationship of bacteriophage to recovery from staphylococcus infections, a controlled study was made during two years on a series of 113 pure staphylococcus infections in which the etiological organism was susceptible to lysis. Of the 89 patients treated, 61 had furuncles, 11 had carbuncles and the remaining had various lesions, mainly abscesses. The results were divided into three categories: those in which convalescence seemed more rapid than average—2 cases; those in which convalescence progressed satisfactorily and similar to recovery under routine treatment—50 cases; and those in which convalescence was not as rapid as might have been expected—37 cases. There was no evidence that bacteriophage administered locally or subcutaneously altered the number of recurrences. Of the treated group, 62 per cent had relapses from 1 to 18 months after bacteriophage therapy. Of the 19 patients who received subcutaneous injections, 13 had recurrences. Bacteriophage had little, if any antiseptic value in closed abscess cavities. Laboratory studies showed that bacteriophage, causing complete sterilization in broth cultures, was markedly inhibited in body fluids and exudates. It is concluded that there is insufficient evidence to regard bacteriophage treatment of staphylococcus infections to be of specific value.

Further Observations on Cutaneous Reactions in Undulant Fever. *J. D. Goldstein*, University of Rochester, Rochester, New York.

Fugitive Wanderings. *Karl F. Meyer*, President of The Society of American Bacteriologists, University of California, San Francisco, California. Guest speaker.

Local Branches of The Society of American Bacteriologists. *H. J. Conn*, Advisor to Local Branches, New York State Agricultural Experiment Station, Geneva, New York.

A Brief Review of the Activities of the Central New York State Branch of The Society of American Bacteriologists. *Ralph P. Tittsler*, University of Rochester, Rochester, New York.

Current Problems and Future Developments in Market Milk Control. *R. S. Breed*, New York State Agricultural Experiment Station, Geneva, New York. Symposium.

ELECTROPHORETIC PHENOMENA OF BACTERIA

II. ELECTROPHORETIC VELOCITIES OF VIRULENT AND NON-VIRULENT C. DIPHTHERIAE

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Electrophoresis has been considered as a probable method of diagnosing, bacteriologically, suspected cases of diphtheria. Data of Jensen and Falk (1928) and their associates, (Jensen *et al.*, 1928; Falk *et al*, 1928) the chief proponents of the method, indicate that under suitable experimental conditions the average velocity of bacilli from virulent cultures is smaller than the average velocity of bacilli from non-virulent cultures. Their investigations led them to believe that the method might be substituted for guinea pig tests for determining the toxigenicity of a given culture of *Corynebacterium diphtheriae*.

To be of use in diagnosing bacteriologically a suspected case of diphtheria, the method of electrophoresis must be capable of returning an unequivocal set of quantitative data, at least as unequivocal as the answer given by the guinea-pig test. When the velocity of the suspected organism is determined the result should be "the organism is toxigenic," or "the organism is non-toxigenic." Can such results be obtained with the technique of electrophoresis employed by the majority of investigators at the present time?

Stone and Weigel (1929) found the method of electrophoresis to be impracticable for routine public health practice. Jones (1931) and Broom and Brown (1932) reported a complete lack of correlation between toxigenicity and velocity. Thompson's data (1931) indicate the existence of a relationship between velocity and virulence, but the two types of bacilli are not sharply sepa-

rated. In view of this fact, Thompson believes the method must await further refinements before it is used for routine work.

We are indebted to the Minnesota State Department of Health for the pure cultures of virulent and avirulent *Corynebacterium diphtheriae* employed in this investigation. The organisms were isolated during the first three months of the year 1934, and their

TABLE 1

Electrophoretic velocities of C. diphtheriae and corresponding virulence tests

CULTURE NUMBER	VIRULENCE	AVERAGE VELOCITY FOR N = 50	CULTURE NUMBER	VIRULENCE	AVERAGE VELOCITY FOR N = 50
		μ/sec./volt/cm.			μ/sec./volt/cm.
1	−	3.1 ±0.3	22	−	1.9 ±0.1
2	−	3.0 ±0.1	23	+	1.8 ±0.1
3	+	2.7 ±0.3	24	+	1.8 ±0.2
4	−	2.6 ±0.2	25	−	1.8 ±0.1
5	−	2.6 ±0.1	26	+	1.8 ±0.1
6	+	2.6 ±0.1	27	−	1.8 ±0.1
7	−	2.5 ±0.1	28	+	1.8 ±0.1
8	−	2.5 ±0.2	29	−	1.8 ±0.1
9	−	2.5 ±0.2	30	+	1.7 ±0.1
10	−	2.4 ±0.1	31	+	1.7 ±0.1
11	−	2.3 ±0.1	32	+	1.7 ±0.1
12	−	2.3 ±0.1	33	+	1.7 ±0.1
13	−	2.2 ±0.1	34	+	1.7 ±0.1
14	+	2.2 ±0.1	35	+	1.7 ±0.1
15	+	2.1 ±0.2	36	+	1.7 ±0.1
16	−	2.1 ±0.1	37	+	1.7 ±0.1
17	−	2.1 ±0.1	38	+	1.6 ±0.1
18	+	2.0 ±0.1	39	+	1.6 ±0.1
19	−	2.0 ±0.1	40	+	1.6 ±0.1
20	+	1.9 ±0.2	41	+	1.5 ±0.1
21	+	1.9 ±0.1	42	+	1.5 ±0.1

The error of each average is measured by $\pm 2 \times$ S.E.$_{\bar{x}}$, where S.E.$_{\bar{x}} = \sigma_x/\sqrt{N}$.

average electrophoretic velocities were immediately determined. The virulence tests reported (guinea-pig inoculations) are the results of the routine tests made with morphologically typical diphtheria bacilli by the State Department of Health.

On receiving these cultures they were plated out on veal-infusion agar, and single colonies were picked. The organisms were then cultivated for five days in 25 cc. of veal-infusion broth (pH

7.6) contained in 125 cc. Erlenmeyer flasks. At the end of this period the growths were sedimented, washed three times with, and suspended in 200 cc. of M/60 phosphate buffer solution, pH 7.3. Fifty measurements were made on each suspension in groups of ten measurements each, a fresh portion of the bacterial suspension being used for each group of determinations. The averages of the fifty measurements were employed in characterizing the

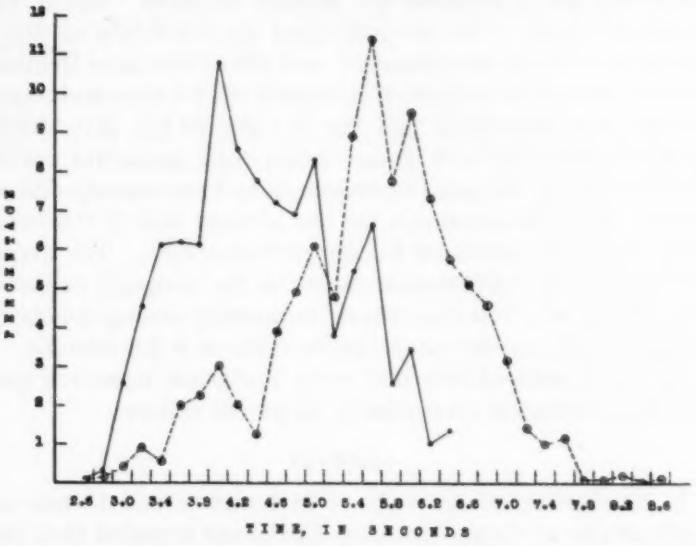

FIG. 1. TIME, IN SECONDS, FOR A GIVEN BACTERIUM TO MIGRATE UNIT DISTANCE

Continuous lines: Measurements on 900 bacilli from 18 avirulent cultures. Broken lines: Measurements on 1200 bacilli from 24 virulent cultures. (*Corynebacterium diphtheriae.*)

electrophoretic velocity of the various strains, using the improved Northrop-Kunitz apparatus (Mudd *et al.*, 1928).

In table 1 are recorded the velocities of these strains in a descending order of magnitude. With this distribution it is impossible to affix a line of demarcation such that organisms below the line shall be considered toxigenic and organisms above the line, non-toxigenic. In other words, there is no sharp line of demarcation between toxigenic and non-toxigenic diphtheria bacilli, and

electrophoretic measurements, as carried out by us, cannot be used to differentiate between the two types of cultures for public health purposes.

However, there is a definite correlation between the velocity and toxigenicity or non-toxigenicity of diphtheria bacilli when similarly diagnosed cultures are considered collectively. Electrophoretic data obtained on a single culture of *C diphtheriae* for public health purposes are perhaps valueless. But if, as is shown in figure 1, all the individual measurements on the 24 toxigenic cultures are assembled and distributed into frequency groups, and the same procedure carried out for the non-toxigenic strains, it is discovered that the two groups fall into distinct classes. Although both groups begin their respective velocity distributions at the same maximum, they have entirely different modes, while the minimum for the virulent cells is 0.6 micron lower than the minimum for the avirulent cells. The average velocity for the 1200 measurements on the toxigenic cultures is 1.8 microns/sec./volt/cm.; the corresponding average for the 900 measurements on the non-toxigenic cultures is 2.2 microns. Of course it is assumed here that every bacterium measured corresponds in biological properties to its parent culture.

SUMMARY

1. An electrophoretic study of 24 toxigenic and 18 non-toxigenic strains of *Corynebacterium diphtheriae* revealed that there is no sharp line of demarcation between the average velocities of the two types of cultures; that the velocities of toxigenic cultures may be distributed among the velocities of non-toxigenic cultures, and *vice versa*.

2. There is, however, a definite degree of correlation between the average electrophoretic velocities of toxigenic and non-toxigenic strains. When the average velocity of all the toxigenic strains studied was compared with the average velocity of all the non-toxigenic strains, it was found that the former velocity is 19.8 per cent slower than the latter.

3. Our findings are in accord with those of Jensen and Falk in so far as we found that, in general, the average velocity of bacilli

in non-toxigenic diphtheria cultures is greater than the average velocity of bacilli in toxigenic diphtheria cultures.

4. The method of electrophoresis cannot, however, be employed, at the present stage of its development, for determining the toxigenicity or non-toxigenicity of a given culture of *Corynebacterium diphtheriae*.

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ELECTROPHORETIC PHENOMENA OF BACTERIA

III. THE ELECTROPHORETIC VELOCITY OF BACTERIA IN RELATION TO GROWTH, SENESCENCE, AND DEATH

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Among the factors which are said to influence the electrophoretic velocity of bacteria is that of the age of the bacteria investigated. However, no definite and designed study has been made of this problem in order to determine to what extent the surface potential difference (P.D.) of bacteria, as measured by their electrophoretic velocity, is influenced by their age. There are a few isolated observations and data in the literature, but these have been made or gathered incidentally in the course of some other study, and are either too limited or too variable and conflicting to be of much service in giving a clear conception of the relationship, if one exists.

Shibley (1924) seems to be the only investigator who has submitted tenable data on the relationship of P.D. to the age of bacteria. He worked with a type I pneumococcus and a strain of *Salmonella schottmuelleri*. His results indicate that young cells (five hours old) carry smaller P.D.'s than cells a few hours older. The P.D. increased to a maximum from the fifth to the tenth or the twelfth hour of growth and then gradually decreased to, and maintained, a somewhat smaller but constant value.

In this paper we report a series of experiments which lead to the conclusion that there is no appreciable change in the electrophoretic velocity of *Escherichia coli* (A19) and *Staphylococcus aureus* (P2) between six hours and ten days of age. The data suggest that only such treatment as will remove or extract a por-

tion of the surface constituents of the cell, or alter their nature by combining with them chemically, will alter the electrophoretic velocity of these strains. The obvious and well-known cases of depression of the electrophoretic velocity by changing the electrolyte content of the suspension medium or by masking the bacterial surface through adsorption of such substances as proteins are excluded. We are concerned here only with the question whether or not a change in surface P.D. occurs during growth, and whether alterations may be produced by aging the cells or treating them in some drastic manner.

THE RELATIONSHIP OF ELECTROPHORETIC VELOCITY TO THE AGE OF ESCH. COLI (A19) AND STAPH. AUREUS (P2)

One of the first considerations in the present investigation was the selection of strains of bacteria which were relatively homogeneous with respect to the velocities of individual cells, and which had narrow ranges when distributed in frequency groups. This would eliminate, to some extent, the error due to one's inability to extract a representative sample from the parent population.

All measurements were carried out with the improved Northrop-Kunitz Microelectrophoresis Apparatus (Mudd, Stewart *et al.*, 1928). Technical details have been given in another paper (as yet unpublished).

Two organisms were used for the study: a strain of *Staph. aureus* (P2), isolated from an abscessed finger, and a strain of *Esch. coli-communior* (A19), a laboratory strain of long standing. Both organisms satisfied the requirement that the velocity range be narrow and the population distribution normal, or relatively so.

The general procedure practised in preparing cultures was as follows: Each organism was inoculated onto a nutrient agar slant and incubated at 37°C. for twenty-four hours. At the end of this period the growths were suspended in 5 cc. of sterile tap water by gently scraping off the growths. A second agar slant was then inoculated, one for each organism, by spreading one loopful of the suspension over its surface, and the slants were incubated for twenty-four hours. This procedure was followed

a third time, after which from 30 to 60 agar slants were inoculated in the same manner. The tubes were stoppered with sterile corks and incubated. Samples were withdrawn for electrophoresis at varying intervals, from six hours to ten days.

Two experiments, with duplicate tests in each, were made employing M/15 phosphate solution, pH 7.3, as the suspension me-

TABLE 1

Summary of data on the relationship of velocity to the age of Esch. coli and Staph. aureus

Figures represent averages of 100 measurements. Cells washed with, and suspended in M/15 phosphate buffer, pH 7.3.

AGE OF CULTURES	ESCH. COLI	STAPH. AUREUS
hours	*microns/sec./volt/cm.*	*microns/sec./volt/cm.*
6	1.8	2.0
9	1.6	2.0
15	1.6	1.9
18	1.6	1.9
21	1.5	1.8
39	1.6	1.8
45	1.5	1.8
69	1.6	1.8
93	1.5	1.8
240	1.5	1.8

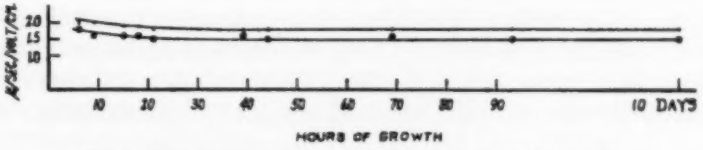

FIG. 1. RELATIONSHIP OF VELOCITY TO AGE OF CULTURE

Upper curve, *Staph. aureus* (P2); lower curve, *B. coli*, (A19). Measurements made in phosphate buffer, M/15, pH 7.3.

dium. The cells were washed three times with the suspension medium, the final sediment being resuspended for measurement. Twenty-five velocity determinations were recorded in each test. The average of the 100 measurements is reported as the value characterizing the velocity of a culture of any given age. The results of the experiments are recorded in table 1, and graphically

in figure 1. They indicate that there is only a slight decrease in velocity of the bacteria from six to two hundred and forty hours of growth. Young cells seem to have greater velocities than old cells.

One series of experiments was carried out with exceedingly old, with heat-killed, and with viable twenty-four hour cultures. All measurements were made on three-times-washed bacilli suspended in distilled water of pH 6.8. Agar slants of *Esch. coli* (A1) were sealed and placed in the incubator on May 16, 1933, and allowed to remain for one hundred and twenty-seven days. Measurements at the end of this period gave an average migration value of 3.5 microns per second per volt per centimeter for a total of 100 readings. Subculturing showed some of these slants to contain viable cells. One thousand measurements on twenty-four-hour cultures of this organism (A1) on May 14, 1933, gave an average velocity of 6.1 microns, while 1000 measurements on heat-killed cells of the same age gave an average value of 5.2.

These results lead to the conclusion that the marked decrease in the average velocity of the aged cells was perhaps due to a thinning out of the bacterial surfaces through disintegration. Thus, the charge per unit area of the cells was decreased. It is unlikely that any appreciable decrease in velocity was produced as a result of exosmosis of salts. Previous experiments had shown the velocity of bacilli in a given suspension to remain relatively constant with time. The heat treatment doubtless brought about its decrease in velocity through coagulation and denaturation of the proteins of the bacterial surfaces (Abramson 1934).

ELECTROPHORETIC VELOCITY OF HEAT-KILLED BACTERIA

Several workers report that they found no significant difference in the velocities of viable and heat-killed bacterial cells. However, this problem has not been extensively studied as the results reported are mainly qualitative.

Winslow, Falk and Caulfield (1923–24) give the results of two of their experiments on heat-killed cells of *Bacillus cereus*, but their data in the two experiments differ to such an extent that we would hesitate to draw any clear-cut conclusion. Their conclu-

sion, however, is that "It is evident that the heat treatment has not materially affected the P.D. on the cells."

We have found that no general statement can be made concerning the effect of heating on the electrophoretic velocity of bacteria. Some strains of a given species will be definitely decreased in their velocity, while others will suffer no measurable change.

Our first experiment on this problem was carried out on a large scale in order to establish clearly the magnitude of the change, if any, and in order to eliminate or balance disturbing factors. *Esch. coli* (A1) was employed as the test organism. Five nutrient agar slants were streaked with a loopful of a distilled water suspension of a twenty-four-hour agar culture of the organism at 8:30 a.m., 10:30 a.m., 12:30 p.m., 2:30 p.m., and 4:30 p.m. These slants were incubated for twenty-four hours and subcultured for two successive days at the above-indicated hours. On the third subculture, 5 agar slants, from the same batch as above, were inoculated from each tube, respectively, and incubated for twenty-four hours. At the end of each of the twenty-four-hour periods the slants were removed, the growth of each slant was suspended in 2 cc. of 0.0001 *N* NaCl solution, the suspensions of each of the five tubes in a series were pooled, and the organisms were washed three times. The sedimented bacteria from the final washing were resuspended in 200 cc. of saline (0.0001 N) and 200 measurements were made in series of 10 measurements each, the cell being flushed out and refilled after each 20 readings. Dichromate cleaning fluid was employed to remove any adsorbed bacteria from the walls of the cell after each 200 measurements.

The measurements on the heat-killed cells were made on three successive days. After the growths were suspended in the saline they were placed in the autoclave for fifteen minutes at 15 pounds pressure. The suspensions were then treated in the same manner as were the viable cells. Figure 2 gives the smoothed frequency distributions for the viable and heat-killed cells.

The mode for the viable cells falls at 1.8 seconds per unit migration, while the mode for the heat-killed cells falls at 2.0 seconds. The corresponding velocities are 6.0 and 5.4 microns

per second per volt per centimeter, respectively. The decrease in velocity produced by heating is 13.7 per cent, considering the average values for the two groups which are 6.1 microns for the viable and 5.2 microns for the heat-killed cells.

Fifteen additional stock cultures of bacteria and yeasts were studied in order to determine whether killing by heat had any influence on their electrophoretic velocity. However, M/120 phosphate buffer, pH 7.3, was employed as the suspension me-

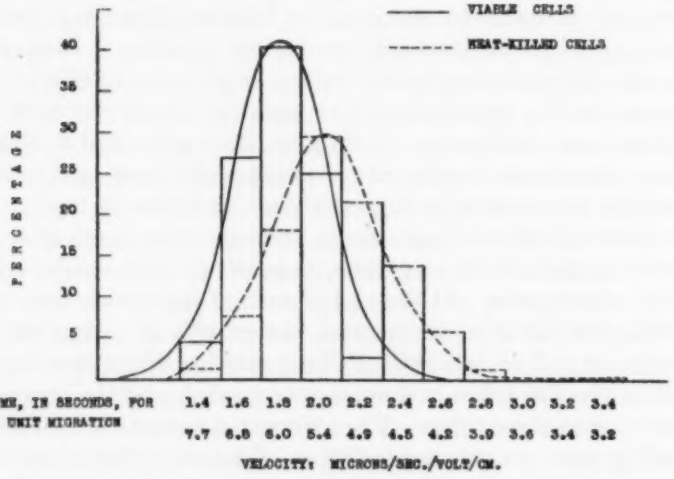

FIG. 2. FREQUENCY DISTRIBUTIONS FOR VIABLE AND HEAT-KILLED CULTURES OF B. COLI (A1)

dium. The experiments were carried out in triplicate, but in some cases the replicated measurements were made on different days. In all cases the replicated measurements represent distinct experiments.

Our procedure in each experiment was as follows: The growths from two agar slants of each organism were suspended in 20 cc. of the buffer mixture and divided into two 10 cc. portions. One portion was autoclaved for fifteen minutes at 15 pounds pressure, after which the two suspensions, viable and heat-killed, were washed three times with the buffer mixture and resuspended for

measurement. From 20 to 50 readings were made on each suspension, depending upon the variation in the individual readings. Table 2 summarizes the results of these experiments.

It is seen from table 2 that 28, 31.4, and 27.6 per cent decreases were obtained in the velocities of *Eberthella typhi*, *Bacillus subtilis*, and *Escherichia coli* (A19), respectively, whereas no appreci-

TABLE 2

Electrophoretic velocity of viable and heat-killed organisms

Cells killed by autoclaving at 15 pounds pressure for 15 minutes. Measurements made in M/120 phosphate buffer solution, pH 7.3.

ORGANISM	VIABLE CELLS				KILLED CELLS			
	Replications			Average	Replications			Average
	0	1	2		0	1	2	
	microns/sec./volt/cm.							
Escherichia coli (-ior) (A12)	1.6	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Escherichia coli (-ior) (A19)	2.9			2.9	2.1			2.1[1]
Eberthella typhi (Pfeiffer)	2.5	2.4	2.5	2.5	1.9	1.8	1.8	1.8[2]
Aerobacter cloaceae (B50a)	1.4	1.3	1.3	1.3	1.2	1.1	1.1	1.1
Pseudomonas aeruginosa	1.7	1.8	1.8	1.8	1.7	1.7	1.7	1.7
Staphylococcus aureus (Lee)	2.0	2.0	2.0	2.0	1.8	1.8	1.7	1.8
Staphylococcus citreus (CC3C)	1.4	1.5	1.6	1.5	1.4	1.4	1.5	1.4
Sarcina lutea (CCC2a)	2.0	2.0	2.1	2.0	2.1	2.1	2.1	2.1
Bacillus subtilis (BB2a)	3.6	3.3	3.6	3.5	2.4	2.4	2.3	2.4[3]
Bacillus megatherium (BB7a)	1.7	1.8	1.7	1.7	1.7	1.8	1.7	1.7
Saccharomyces cerevisiae (Y1a)	0	0	0	0	0	0	0	0
Torula cremoris (Y26a)	0.5	0.5	0	0.5	0	0	0	0
Pink yeast from oyster (Y25a)	2.5	2.6	2.7	2.6	0.8	0.8	0.8	0.8[4]
Pink yeast from oyster (206)	2.6	2.6	2.9	2.7	0.8	0.8	0.8	0.8[5]
Pink yeast from oyster (208)	2.3	2.6	2.5	2.5	0.8	0.8	0.8	0.8[6]

Percentage decreases in velocity: [1] 27.6 per cent; [2] 28 per cent; [3] 31.4 per cent; [4] 69.3 per cent; [5] 70.4 per cent; [6] 68 per cent.

able decrease was produced in the velocities of the other bacteria studied. *Saccharomyces cerevisiae* showed practically no motion in the viable or killed condition even when 120 volts pressure was applied to the zinc electrodes of the electrophoresis apparatus. *Torula cremoris* decreased from 0.5 micron to zero velocity, while the three strains of pink yeasts showed decreases amounting to from 68 to 70.4 per cent. Treatment of these pink yeasts with

phenol, formalin, and mercuric chloride for one hour at 37°C. produced no noticeable effect, whereas extraction with hot or cold alcohol produced decreases of the order of magnitude reported in table 2.

One very interesting observation on the behavior of these pink yeasts when heated is the persistent flocculation of yeast 206, while Y25a and 208 remain perfectly stable. When heated in a water bath at 60°C., or in the autoclave at 15 pounds pressure for 15 minutes, the cells of 206 become completely sedimented, the suspension medium being perfectly clear. This sedimentation on heating takes place in distilled water, weak solutions of electrolytes, and in M/60 phosphate buffer.

The flocculation of yeast 206 indicates that the P.D. is perhaps the major factor in its stability, whereas P.D. may be a minor factor in the stability of the other two strains. Mudd (1933) has discussed the problem of the relative importance of charge or P.D., work of cohesion, and surface hydration to the stability of bacterial suspensions to some length in his lecture on agglutination at the "First Cold Spring Harbor Symposium On Quantitative Biology."

ELECTROPHORETIC VELOCITY OF CELLS EXTRACTED WITH 70 PER CENT ALCOHOL

Well defined decreases were noted in the velocities of a strain of *Esch. coli*, a strain of *Staph. aureus*, and the pink yeasts recorded in table 2. There was a 70 per cent decrease in the velocity of the yeast cells.

The organisms were grown for twenty-four hours on nutrient agar slants. The growths were suspended in 5 cc. of 70 per cent alcohol and heated in a water bath to 70°C. for one hour. After heating, the cells were washed three times with, and suspended in M/120 phosphate buffer, pH 7.3. Table 3 gives the results of these experiments.

Three experiments were performed in testing the effect of alcohol on the cells, while two were carried out for the controls. The percentage decrease for *Esch. coli* is 19.3. The decrease for *Staph. aureus* is 27.6 per cent. Fifty velocity readings were made on each suspension.

TABLE 3

Electrophoretic velocity of Esch. coli (A19) and Staph. aureus (P2) killed by heating in 70 per cent alcohol for one hour at 70°C.

Cells washed three times and suspended in M/120 phosphate buffer, pH 7.3

Velocity in microns/sec./volt/cm.

UNTREATED CELLS		KILLED WITH HOT ALCOHOL	
2.5 2.6	2.6	2.2 2.1 2.0	2.1
2.9 2.8	2.9	2 1 2.1 2 1	2.1

TABLE 4

Electrophoretic velocity of cells treated with formalin, phenol, and mercuric chloride for one hour at 37°C.

Measurements made in phosphate buffer solution, M/120 at pH 7.3

REPLICATIONS	FORMALIN, 2 PER CENT	MERCURIC CHLORIDE, 0.1 PER CENT	PHENOL, 5 PER CENT	CONTROLS
	microns/sec./volt/cm.	*microns/sec./volt/cm.*	*microns/sec./volt/cm.*	*microns/sec./volt/cm.*
Pink Yeast (Y25a)				
0	3.1	3.3	3.1	3.1
1	3.1	3.0	3.0	3.0
2	3.1	3.1	3.1	3.1
Average	3.1	3.1	3.1	3.1
Esch. coli (A19)				
0	2.7	2.9	2.6	2.9
1	2.8	2.8	2.9	2.8
2	2.9	2.9	2.6	2.8
Average	2.8	2.9	2.7	2.8
Staph. aureus (P2)				
0	2.0	2.1	2.1	2.3
1	2.0	2.1	2.1	2.0
2	2.0	2.3	1.7	1.9
Average	2.0	2.2	2.0	2.1

ELECTROPHORETIC VELOCITY OF CELLS TREATED WITH FORMALIN, PHENOL, AND MERCURIC CHLORIDE

Tomita (1925–26) seems to be the only worker who has studied this problem and presented data on it. However, Tomita's technique and the behavior of particles in his electrophoresis cell appear to invalidate his results.

In our work a pink yeast (Y25a), *Esch. coli* (A19), and *Staph. aureus* (P2) were treated in the following manner: The growths from four nutrient agar slants of each organism (Sabouraud's agar for the yeast) were removed by washing them off with 10 cc. of (1) 5 per cent phenol; (2) 2 per cent formalin; (3) 0.1 per cent mercuric chloride; and (4) M/120 phosphate buffer, pH 7.3, respectively. The suspensions were then placed in the incubator at 37°C. for one hour. The cells of each suspension were then washed three times with and suspended in, phosphate buffer, M/120, pH 7.3. Each experiment was performed three times. Table 4 shows that no significant change was produced in the respective velocities of the organisms by treating them with these destructive agents.

SUMMARY AND CONCLUSIONS

1. Electrophoretic data obtained with strains of *Escherichia coli* and *Staphylococcus aureus* lead to the conclusion that the velocity of these bacteria does not vary with the age of the culture, except when disintegrating cells are considered. It is shown that the velocities of these organisms are relatively constant from about six hours of growth up to ten days.

2. The velocity of heat-killed bacteria may or may not be the same as that for the bacteria in the viable condition. Some strains of a given species may be lowered in velocity, while other strains will suffer no measurable change.

3. Three strains of pink yeasts showed decreases in their velocities amounting to from 68 to 70 per cent when the cells were heat-killed.

4. Extraction of a strain each of *Escherichia coli*, *Staphlococcus aureus* and a pink yeast with hot 70 per cent alcohol lowered their

respective velocities approximately to those values found when the cells were killed in phosphate buffer.

5. Treatment of *Escherichia coli*, *Staphylococcus aureus*, and a pink yeast with formalin, phenol, and mercuric chloride for one hour at incubator temperature produced no measurable change in the velocities of the cells.

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A SEROLOGICAL VARIANT OF SALMONELLA AERTRYCKE ISOLATED FROM PIGEONS[1]

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The occurrence of *Salmonella aertrycke* in diseases of pigeons has been noted by a number of workers. Recently Jungherr and Wilcox (1934) and Black (1935) have reported the isolation from pigeons of variants of *S. aertrycke* which were atypical in their action on maltose. Among cultures isolated from the same flock were found organisms which caused a prompt production of acid and gas from the sugar, organisms which produced a slight acidity after prolonged incubation, and organisms which apparently did not ferment maltose.

The writer has isolated organisms closely resembling the strains of Jungherr and Wilcox and of Black from pigeons. Unfortunately, only two cultures of paratyphoid bacilli were isolated from the infected flock, since no further specimens were submitted. It was also impossible to obtain any history of the disease in this flock. The two pigeons from which paratyphoid bacilli were isolated had died of the disease. They were greatly emaciated and were said by the owner to have been ill for some time. Petechial hemorrhages and congested areas were observed on the hearts and multiple small white areas of degeneration in the livers. Paratyphoid bacilli were isolated from the heart, liver and spleen of both birds.

The present paper is a report of a biochemical and serological study of the cultures isolated by the writer, and of cultures obtained through the courtesy of Dr. Jungherr and Dr. Black.

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

MATERIALS AND METHODS

Following is a list of cultures used in the work, their source and date of isolation:

19500—Isolated from adult pigeon, 1934.

19525—Isolated from adult pigeon, 1934.

N. J. 4015—Received from Dr. J. J. Black, New Jersey Agricultural Experiment Station. Isolated from heart blood of pigeon, 1934.

N. J. 4315—Received from Dr. J. J. Black. Isolated from heart blood of pigeon, 1934.

Storrs 5845—Received from Dr. E. Jungherr, Connecticut Agricultural Experiment Station. Isolated from squab.

Storrs 5899—Received from Dr. E. Jungherr. Isolated from squab.

OW—*S. aetrycke*, Ovum W. Received from Dr. L. F. Rettger. Isolated from ovary of duck, 1919.

17666—*S. aertrycke*, 17666. Isolated from liver of foal, 1932.

NMI—*S. aertrycke*, NMI. Isolated from liver of mouse, 1935.

NGP2—*S. aertrycke*, NGP2. Isolated from liver of guinea pig, 1935.

WH_2—*S. abortus-equi*, WH_2. Isolated from aborted equine fetus, 1932.

4K88—*S. abortus-equi*, 4K88. Received from Maj. F. H. K. Reynolds, Army Medical Center. Isolated from aborted equine fetus, 1934.

15746—*S. pullorum* 15746. Isolated from baby chick, 1928.

In examining the serological properties of the organisms, formalinized infusion-broth cultures were used as antigens in the test for floccular agglutination. The tests for somatic agglutinins were carried out with antigens prepared by the method of Gardner (1929). In agglutinin absorption tests the method of Edwards and Rettger (1927) was used. Agglutination tests were held at 52°C. for four hours and in the icebox overnight. They were read at the end of the four-hour incubation period and a final reading taken the following morning.

Biochemical properties. All the strains from pigeons yielded a negative test in the rhamnose medium of Bitter, Weigman and

Habs (1926). With the exception of their behavior in the Bitter test and their action on maltose the organisms possessed the biochemical characters attributed to *S. aertrycke*. They fermented glucose, arabinose, rhamnose, xylose, trehalose, dulcitol, inositol and mannitol with the production of acid and gas. Lactose and sucrose were not attacked. The organisms produced hydrogen sulfide and failed to form indol.

The action of the different strains on maltose varied. Cultures 19525 and Storrs 5899 produced acid and gas from maltose after twenty-four hours incubation. Cultures 19500, N. J. 4015, N. J. 4315, and Storrs 5845 produced a barely perceptible acidity in one per cent maltose broth after overnight incubation. This acidity disappeared during the second twenty-four hours of incubation and the broth remained neutral or alkaline during the remainder of the fourteen day period of observation. An increase in the amount of maltose to 4 per cent resulted in a marked increase in the amount of acid produced by all the cultures and a slight amount of gas production by some of them. Stock cultures of *Salmonella pullorum* also produced an easily perceptible amount of acid in twenty-four hours in the broth containing 4 per cent maltose. These results indicate that the early transient acid production of the pigeon cultures is due to impurity of the sugar. A second sample of maltose was tested in the same way and the production of acid was much less than with the first lot of sugar. The maltose was sterilized by filtration through clean, freshly burned filters in all tests. Jungherr and Wilcox (1934) reported that the culture Storrs 5845 did not ferment maltose. Black (1935) reported that cultures N. J. 4015 and N. J. 4315 produced a slight acidity after twenty-four hours incubation in maltose broth. This reaction disappeared after 48 hours. From the results obtained here it seems probably that these four strains do not ferment maltose.

Serological characters. Jungherr and Wilcox (1934) reported that the cultures which they isolated were serologically identical with *S. aertrycke*. They examined the somatic antigens by simple agglutination and the flagellar antigens by agglutinin absorption.

In the present work it has been found that all the pigeon cul-

tures possess flocculating antigens identical with those of *S. aertrycke*. These cultures were able to exhaust *S. aertrycke* antiserums of flocculating agglutinins for the homologous organisms. Likewise stock cultures of *S. aertrycke* exhausted the antiserums of cultures 19500 and 19525 of flocculating agglutinins for their homologous strains.

TABLE 1

Somatic antigens of pigeon paratyphoid absorption tests with S. aertrycke antiserum

ANTIGENS	S. AERTRYCKE 17666 ANTISERUM ABSORBED BY								
	19500	19525	N. J. 4015	N. J. 4315	STORRS 5899	S. ABORTUS EQUI WH_2	S. ABORTUS EQUI 4K88	S. AERTRYCKE OW	S. AERTRYCKE NMI
19500	0*	0	0	0	0	0	0	0	0
19525	0	0	0	0	0	0	0	0	0
N. J. 4015	0	0	0	0	0	0	0	0	0
N. J. 4315	0	0	0	0	0	0	0	0	0
Storrs 5845	0	0	0	0	0	0	0	0	0
Storrs 5899	0	0	0	0	0	0	0	0	0
S. abortus-equi WH_2	0	0	0	0	0	0	0	0	0
S. abortus-equi 4K88	0	0	0	0	0	0	0	0	0
S. aertrycke OW	500	1,000	1,000	1,000	1,000	500	1,000	0	0
S. aertrycke NMI	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0	0
S. aertrycke NGP2	1,000	500	1,000	1,000	1,000	500	500	0	0
S. aertrycke 17666	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0	0

* Figures indicate highest dilution at which agglutination occurred.
0 indicates no agglutination at 1:50.

In performing the agglutination tests in which the alcoholic antigens of Gardner (1929) were used, *S. pullorum* antiserum was included in the tests since Jungherr and Wilcox (1934) emphasized the agglutination of their pigeon strains with *S. pullorum* antiserum. In these tests cross agglutination between *S. pullorum* and the pigeon strains occurred only in low dilutions, not exceeding 10 per cent of the titers of the serums. In other respects the results of the tests were quite similar to those published by

Jungherr and Wilcox, the pigeon strains and *S. aertrycke* cross agglutinating in high dilution. While the results were irregular in some particulars, in general the pigeon strains seemed to be somewhat more easily agglutinable than the *S. aertrycke* cultures, and the strains of *Salmonella abortus-equi* quite closely paralleled the pigeon strains in their reactions. However the differences were not sufficiently pronounced to lead to the conclusion that antigenic differences existed in the somatic complexes of the strains.

When the somatic characters of the organisms were examined by agglutinin absorption striking differences were observed. *S. aertrycke*, *S. abortus-equi* and *Salmonella* (Derby) were able to exhaust completely the somatic agglutinins from antiserums prepared from two pigeon strains. The results obtained using *S. aertrycke* antiserum are given in table 1. It is evident that *S. abortus-equi* removed all somatic agglutinins active on the pigeon strains from *S. aertrycke* antiserum. It is likewise evident that while the pigeon strains removed the agglutinins for *S. abortus-equi* from *S. aertrycke* serum, they failed to exhaust the serum of agglutinins for *S. aertrycke*. Although the absorbing doses of the pigeon strains were increased to 40 plate cultures per cubic centimeter of serum, they were unable to exhaust the somatic agglutinins from *S. aertrycke* antiserum. It seems obvious, therefore, that the pigeon strains possess the somatic complex of *S. abortus-equi* and not that of *S. aertrycke*.

DISCUSSION

The present status of methods of differentiating the species of the genus *Salmonella* is so well known and has been so widely discussed as to need no comment. The available knowledge concerning the serological relationships of these bacilli has been summarized recently in the report of the Salmonella Sub-committee of the Nomenclature Committee of the International Society for Microbiology (1934). The work reported here adds still another sub-species or variety to the fast growing list of serological types within the genus. Since the variants studied here were first described by Jungherr and Wilcox at the Connecti-

cut Agricultural Experiment Station, the designation *S. aertrycke* var. *Storrs* is suggested. This is in keeping with the recommendation of the above named committee that sub-species or varieties be designated by the place of their original isolation. The antigenic formula of the variety is as follows:

SOMATIC ANTIGEN	FLOCCULATING ANTIGEN	
	Specific	Non-specific
IV	i	1,2,3

Since the variety here described is distinguished only by the absence of an antigenic factor in its somatic complex, and since it is the somatic antigens which are affected by roughness, it is necessary to demonstrate that the cultures used in these studies were not rough variants. All the strains used in the work were subjected to the tests suggested by White (1926) for roughness and to the saline agglutination test of Wilson (1930) and the thermo-agglutination test of Wilson (1933). In addition, the acriflavine method of Pampana (1933) was applied to the cultures. In all of these procedures the strains under study reacted as normal smooth forms. Very recently isolated strains were included in the study, also, to rule out further the possibility that the differences observed might be due to the use of rough variants. In addition to the antiserum mentioned in the table other antiserums derived from *S. aertrycke* were studied. The results obtained with these were identical with the results given in the tables. The tests have been repeated and constant results obtained.

For several years the specific flocculating antigen of *S. aertrycke* was known to exist only in association with the somatic complex of the *S. aertrycke—Salmonella Schottmülleri* group. Recently Smith (1934) demonstrated that it may be found in *Salmonella* (Aberdeen). The finding of the specific flocculating antigen of *S. aertrycke* in combination with the somatic antigen of the *S. abortus-equi—Salmonella* (Reading)—*Salmonella* (Derby) group suggests that this flocculating antigen may be much more widely distributed throughout the genus than has been suspected. This

adds weight to the theory of White (1926) that the salmonellas are a series of loss variants and that *S. aertrycke* is one of the most primitive representatives.

Since the variant described here has been isolated from pigeons in three widely separated communities, it is desirable that other cultures from pigeons should be examined to determine whether they are members of this type.

SUMMARY

A serological variant of *Salmonella aertrycke* is described. The organism was isolated from pigeons in three widely separated communities. It differs from *Salmonella aertrycke* in that it possesses somatic antigens identical with those of *Salmonella arbortus-equi*. Some strains of the variant fail to ferment maltose and all give a negative Bitter test. The term *Salmonella aertrycke* var. *Storrs* is proposed to designate this type.

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THE INFLUENCE OF HEAT AND STORAGE ON THE ELECTROPHORETIC MIGRATION VELOCITIES OF VARIOUS MICROÖRGANISMS

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Although the literature contains numerous references to the effect of heat and storage on the bacterial cell, a careful review fails to reveal any information as to the influence of either on the electrophoretic migration velocities. Recent workers, in their studies of those physical agents which influence the zeta potential, suggest that heat may play a part, but experimental evidence for this has not, to our knowedge, been published.

In the various studies on the effect of x-rays and radium on the zeta potential (Dozois, Titsler, Lisse and Davey (1932), and Dozois, Ward and Hachtel (1935)) the question has arisen of the part heat may play in the results obtained. In the determination of the zeta potential of pathogenic microörganisms, using the Kunitz modification of the Northrop-Kunitz microcataphoresis cell (1928)—work now under way by the authors—it was deemed advisable to determine whether or not the microörganisms could first be killed by heating before measurements were made. If heating the microörganisms to a temperature sufficiently high to kill them did not alter their zeta potential or interfere with the final results, then this procedure could be followed.

In making studies on the zeta potential of microörganisms it is also necessary to have information as to the effect of storage on the suspensions prepared for observation. Dozois (1932) in his studies has verified to some extent the findings of Shibley, (1924) that the quantity of the zeta potential varies until the culture is eighteen hours old, after which it remains constant.

METHOD

Preparation of suspensions. The cultures of *Escherichia coli*, *Aerobacter aerogenes* and *Corynebacterium diphtheriae* (*gravis*, *mitis*, and intermediate) which were used in this investigation have been tested in previous studies and found to give constant migration velocities. The cultures of *Escherichia coli* and *Aerobacter aerogenes* were grown on proteose peptone agar slants, pH 6.8, for twenty-four hours at 37°C. The cultures of *Corynebacterium diphtheriae* were grown on Löffler's serum medium, pH 7.0, for the same length of time. The growth was washed off the surface with distilled water and then filtered through cotton and centrifugalized at about 2700 r.p.m. for forty-five minutes. The supernatant fluid was then removed and the cells resuspended and rewashed twice. The suspensions were then filtered through filter paper and diluted as found necessary. Measurements of the electrophoretic migration velocities were made on each suspension and these readings used as controls. The suspensions were then heated in a water-bath at the temperatures shown in the tables and measurements made at the time-intervals indicated. The stored suspensions were placed in the ice box or held at room-temperature as shown in the tables.

The growths of *Escherichia coli* and *Aerobacter aerogenes* were washed and suspended in distilled water of a pH 6.8. For *Corynebacterium diphtheriae* distilled water of pH 7.0 was used. A pH determination was made on each suspension before and after heating or storage. In those instances where measurements did not follow immediately after treatment a determination was also made just previous to the electrophoretic measurement. In each case the pH was found to remain unchanged. A determination made on half of the suspensions immediately following the electrophoretic migration velocity measurements showed similar results.

For measurements of the zeta potential, the Kunitz modification of the Northrop-Kunitz microcataphoresis cell (1928) was used with a micro lamp, the light from which was passed through a solution of ammoniacal copper sulphate. A Bausch and Lomb

TABLE 1

Effect of storage at 5°C. on electrophoretic migration velocities

STORAGE AT 5°C.	ELECTROPHORETIC MIGRATION VELOCITIES, SECONDS PER 0.5 MM.				
	Bacterial suspensions				
	E. coli	*A. aerogenes*	*C. diphtheriae*		
			Gravis	Mitis	Intermed.
hours					
Control	13.2	14.6	10.0	8.4	8.8
1	13.3	14.5	10.0	8.3	8.8
2	13.5	14.6	10.0	8.2	8.8
3	13.1	14.5	10.1	8.4	8.9
4	13.5	14.5	10.1	8.4	8.8
5	13.0	14.5	10.1	8.4	8.7
6	13.4	14.5	10.1	8.3	8.8
8	13.3	14.6	10.0	8.4	8.7
10	13.2	14.6	9.9	8.3	8 7
12	13.2	14.6	10.0	8.3	8.7
18	13.5	14.6	10.1	8.4	8.7
24	13.4	14 6	10.0	8.4	8.7
36	13.3	14.6	10.1	8.3	8.8
48	13.3	14.6	10.0	8.4	8.7

TABLE 2

Effect of storage at room temperature on electrophoretic migration velocities

STORAGE AT 27°C.	ELECTROPHORETIC MIGRATION VELOCITIES, SECONDS PER 0.5 MM.				
	Bacterial suspensions				
	E. coli	*A. aerogenes*	*C. diphtheriae*		
			Gravis	Mitis	Intermed.
hours					
Control	13.3	14.5	10.1	8.3	8.8
1	13.2	14.5	10.2	8.3	8.9
2	13.2	14.6	10.1	8.4	9.0
3	13.4	14.7	10.3	8.4	9.1
4	13.6	14.9	10.4	8.4	9.0
5	13.7	14.8	10.5	8.4	9.1
6	13.8	14.9	10.5	8.5	9.1
8	13.7	14.9	10.6	8.6	9.2

8 mm., 0.50 n.a. 21× objective and a 10× eye piece was employed. Twenty readings were made at the two stationary levels, i.e., 0.21 and 0.79 of the inside depth of the cell. An applied

potential of 118 volts was used. This applied voltage gave a potential drop through the cell of 2.5 volts. The electrophoretic mobilities are given in terms of seconds per 0.5 mm. per 118 volts.

TABLE 3

Effect of heat on electrophoretic migration velocities

TEMPERATURE	ELECTROPHORETIC MIGRATION VELOCITIES, SECONDS PER 0.5 MM.				
	Bacterial suspensions				
	E. coli	*A. aerogenes*	*C. diphtheriae*		
			Gravis	Mitis	Intermed.
degrees					
Control	13.3	14.5	9.7	8.4	8.9
60	13.4	14.5	9.7	8 3	9.1
70	13.4	14.6	9.8	8.5	9.0
75	13.6	14.7	9.9	8.6	9.2
80	13.6	14.9	10.0	8.6	9.2
85	13.7	14.9	9.9	8.7	9.3
90	14.0	15.0	10.1	8.9	9.4
95	13.9	15.1	10.2	8.8	9.6

TABLE 4

Effect of storage at 27°C. on electrophoretic migration velocities of bacterial suspensions heated to 60°C. for thirty minutes

STORAGE AT 27°C.	ELECTROPHORETIC MIGRATION VELOCITIES, SECONDS PER 0.5 MM.				
	Bacterial suspensions				
	E. coli	*A. aerogenes*	*C. diphtheriae*		
			Gravis	Mitis	Intermed.
hours					
Control*	13.3	14.5	9.7	8.4	8.9
Control†	13.4	14.5	9.7	8.3	9.1
1	13.4	14.6	9.7	8.4	8.9
2	13.5	14.7	9.9	8.5	9.1
3	13.6	14.7	9.9	8.6	9.2
5	13.6	14.8	9.9	8.6	9.3
8	13.7	14.9	10.0	8.7	9.5

* Before heating.
† After heating.

SUMMARY

1. Observations made on each bacterial suspension before the electrophoretic migration velocity was determined show that

storage and heat, under the conditions observed, did not alter the pH which remained at 6.8 for the *Escherichia coli* and *Aerobacter aerogenes* and at 7.0 for the *Corynebacterim diphtheriae*.

2. The bacterial suspensions studied were kept at a temperature of 5°C. for as long as forty-eight hours without significant alteration in the zeta potential.

3. The bacterial suspensions studied were kept at 27°C. for two hours without a significant change, after which time a measureable but not marked alteration in the zeta potential took place.

4. The bacterial suspensions studied were heated at a temperature of 60°C. for thirty minutes with no significant change in the zeta potential.

5. At temperatures above 60°C. there was a slight tendency toward a decrease in the zeta potential. This decrease became more marked as the temperature was increased.

6. The electrophoretic migration velocity of bacterial suspensions heated to a temperature of 60°C. for thirty minutes remained fairly constant for two hours at 27°C. After this time a slight but gradual decrease in the electrophoretic migration velocity occurred.

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A COMPARISON OF THE EIJKMAN TEST WITH OTHER TESTS FOR DETERMINING ESCHERICHIA COLI IN SEWAGE

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The routine use of a Modified Eijkman method in the examination of oysters, crabmeat, and other substances has been discussed by the authors (1935). Only three sewage samples were reported, a number obviously insufficient from which to draw any conclusion, especially since these observations were made before certain improvements in the Eijkman method had been applied. Therefore, it was thought desirable to study the method further with samples of raw sewage, effluent and sludge in order to evaluate more accurately the authors' modification of the Eijkman test for this purpose.

Brown and Skinner (1930) have criticized the value of the test based on pure cultures, alone. In the present investigation, the dilution method, using triplicate inoculations for each dilution, has been used to compare the Eijkman test with other tests used for the examination of water in different parts of America and with the Standard lactose broth method.

PROCEDURE

High dilutions of sewage were made in order to insure that the last two or three dilutions would be negative, a necessary step in order to calculate most probable numbers with accuracy. All cultures were incubated at 37°C., for approximately twenty-four and forty-eight hours, except the Eijkman tubes which were incubated at 46°C. Eosin-methylene-blue (E.M.B.) agar plates were streaked from tubes and after twenty-four and forty-eight

hours incubation, one or two typical colonies from each plate were inoculated into citrate, Eijkman and lactose broth for confirmation. Cultures which produced typical colonies on E.M.B. plates and formed gas in lactose and Eijkman broths but which were unable to grow in the citrate medium of Koser (1923) were considered as *Escherichia coli*.

MEDIA USED IN THE COMPARISON

Lactose broth and eosin-methylene-blue agar were prepared according to Standard Methods (1933). The buffered lactose broth was of the formula outlined in the non-standard section.

Salle's crystal violet lactose broth (1930), Dominick-Lauter's brom-cresol-purple, methylene blue, lactose broth (Standard Methods, 7th edition), Ritter's basic fuchsin, lactose broth (1932) and Stark and England's formate ricinoleate broth (1935) were prepared according to the directions of the authors. The formula for the modified Eijkman medium of Perry and Hajna (1935), is, in the final concentration, as follows:

	per cent
Bacto-peptone	1.5
Bacto-dextrose	0.3
K_2HPO_4	0.4
KH_2PO_4	0.15
NaCl	0.5

No titration nor filtration is required. The final pH is 6.9.

RESULTS

The results obtained from the examination of ten samples of raw sewage for *Escherichia coli* are given in table 1. Two hundred and seventy tubes of each medium were inoculated. Salle's (crystal-violet lactose broth) had the smallest number (171) of tubes showing gas fermentation. Three more (174) of the modified Eijkman broth tubes had gas. The greatest number (185) of tubes with gas was obtained with Standard lactose broth. Eijkman broth, however, although with next to the smallest number of tubes showing gas, yielded the greatest number (161) of confirmatory tests for *Escherichia coli*. Salle's medium yielded

less than half as many (80) confirmations. Standard lactose broth yielded almost as many (153) confirmations for *Escherichia coli*, but due to gas production by bacteria other than *Escherichia coli* only 82.8 per cent of the tubes with gas confirmed as compared

TABLE 1

Comparative results, both presumptive and confirmed, for Escherichia coli and coli-aerogenes organisms in raw sewage with various media and with Eijkman broth

Number of Samples Examined—10

	STANDARD LACTOSE	STANDARD LACTOSE PLUS K_2HPO_4	EIJKMAN	RITTER'S	SALLE'S	DOMINICK-LAUTER'S	STARK AND ENGLAND'S
Total tubes inoculated	270	270	270	270	270	270	270
Tubes having gas at 24 and 48 hours	185	181	174	177	171	178	183
(a) Tubes having gas at 24 hours	182	180	170	176	155	176	183
(b) Tubes having gas at 48 hours	3	1	4	1	16	2	0
Total confirmations, coli-aerogenes group	182	180	171	176	171	178	183
(a) Number of 24-hours gas tubes confirmed	181	179	167	176	155	176	183
(b) Number of 48-hour gas tubes confirmed	1	1	4	0	16	2	0
Total confirmations, *Esch. coli*	153	105	161	141	80	155	148
(a) Number of 24-hour gas tubes confirmed	152	105	157	141	76	155	148
(b) Number of 48-hour gas tubes confirmed	1	0	4	0	4	0	0
Per cent of tubes with fermentation at 24 and 48 hours	68.5	67.0	63.3	65.5	63.3	65.9	67.8
Per cent of inoculated tubes confirmed for *Esch. coli*	56.7	39.0	59.6	52.2	29.6	57.4	54.9
Per cent of gas tubes confirmed for *Esch. coli*	82.8	58.0	92.5	79.8	46.8	87.0	81.0

with 92.5 per cent of the positive Eijkman tubes. These results indicate that the modified Eijkman method is superior to the other methods for the isolation of *Escherichia coli*.

In the broths containing bacteriostatic dyes, there seems to be an inhibiting effect upon *Escherichia coli*. Brom-cresol-purple

methylene-blue broth seems to be the best of the group. The failure of crystal-violet lactose and buffered lactose broths to detect greater numbers of *Escherichia coli* seems to lie in the buffering action of the two media, which favors the *Aerobacter aerogenes* and other members of the colon group over *Escherichia coli* at 37°C.

TABLE 2

Comparative results, both presumptive and confirmed, for Escherichia coli and coli-aerogenes organisms in Eijkman medium and in standard lactose broth

	20 SAMPLES OF RAW SEWAGE		4 SAMPLES OF EFFLUENT		10 SAMPLES OF SLUDGE	
	L	E	L	E	L	E
Total tubes inoculated	624	624	102	102	270	270
Tubes having gas at 24 and 48 hours	370	395	78	45	165	142
(a) Tubes having gas at 24 hours	362	373	66	38	144	121
(b) Tubes having gas at 48 hours	8	22	12	7	21	21
Total confirmations, coli-aerogenes group	365	389	78	39	145	119
(a) Number of 24-hour gas tubes confirmed	360	369	66	37	141	118
(b) Number of 48-hour gas tubes confirmed	5	20	12	2	4	1
Total confirmations, *Escherichia coli*	320	373	16	28	129	117
(a) Number of 24-hour gas tubes confirmed	316	356	13	28	128	117
(b) Number of 48-hour gas tubes confirmed	4	17	3	0	1	0
Per cent of tubes with fermentation at 24 and 48 hours	59.3	63.3	76.5	44.0	61.2	52.6
Per cent of inoculated tubes confirmed for *Escherichia coli*	51.3	59.8	15.7	27.5	47.8	43.3
Per cent of gas tubes confirmed for *Escherichia coli*	87.8	95.8	20.5	62.3	78.3	82.4

Dominick-Lauter's, Stark and England's and Salle's media gave no false presumptives. Ritter's medium and buffered lactose broth each had one false presumptive.

A further comparison of results obtained with twenty samples of raw sewage, four of sewage effluent and ten of sludge, by Standard lactose broth and Eijkman medium is given in table 2.

Of 624 tubes of lactose broth and a like number of Eijkman broth tubes inoculated with raw sewage, 395 or 63.3 per cent of the Eijkman tubes and 370 or 59.3 per cent of the lactose tubes showed gas. It is interesting to note that 95.8 per cent of the positive Eijkman tubes were confirmed for *Escherichia coli* as compared with 87.8 per cent of the lactose broth tubes, thus demonstrating not only the greater efficiency of the Eijkman test for isolation of *Escherichia coli* but also demonstrating that, in these samples of raw sewage, more tubes (373) of the Eijkman broth were confirmed for *Escherichia coli* than could be confirmed (365) for the entire colon group by Standard lactose broth. It is important that this predominance of *Escherichia coli* in raw sewage be carefully considered for it is of the utmost significance in considering the relative merits of *Escherichia coli* as opposed to the use of the entire group as an index of fecal pollution.

Far less of the tubes of both lactose and Eijkman broth inoculated with sewage effluent in which gas fermentation occurred could be confirmed for *Escherichia coli* than in the case of those inoculated with raw sewage. Only 20.5 per cent of the positive lactose broth tubes could be confirmed against 62.3 per cent of the Eijkman tubes. It is interesting to note the ratio of *Escherichia coli* (Eijkman method) to the colon group (Standard lactose broth) for raw sewage and for sewage effluent, which in raw sewage was 1.03 to 1.0 while for sewage effluent it was 1 to 2.8. *Escherichia coli* was found to predominate therefore, in raw sewage but other members of the colon group either outgrew or outlived *Escherichia coli* in the effluent. In sewage sludge *Escherichia coli* tends, apparently, to regain its original relative position for the ratio was found to be approximately 1 to 1.2. More tubes (129 vs. 117) of the lactose broth than of the Eijkman broth inoculated with sewage sludge were confirmed for *Escherichia coli*. The percent of positive gas tubes, however, which confirmed was greater for the Eijkman method (78.3 vs. 82.4). The numbers of samples of both sewage effluent and sludge were small (4 and 10 respectively).

SUMMARY

Certain media which have been proposed as possible substitutes for Standard lactose broth have been compared with the authors' modified Eijkman medium for relative efficiency in the isolation of *Escherichia coli* from raw sewage. The Eijkman test was found superior to Standard lactose broth, buffered lactose broth, Ritter's, Salle's, Dominick-Lauter's, and Stark's and England's broths both in the number of isolations of *Escherichia coli* made and in the number of gas tubes confirmed for *Escherichia coli*.

The relative efficiency of Standard lactose broth and the modified Eijkman broth of the authors for the isolation of *Escherichia coli* from raw sewage, sewage effluent and sludge was tested. *Escherichia coli* was recovered from more of the tubes of Eijkman broth than of lactose except in the case of sewage sludge. Four and a half per cent more of the lactose broth tubes inoculated with sludge confirmed for *Escherichia coli*. A much larger percentage of the Eijkman tubes with gas confirmed in all instances.

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STUDIES ON THE CULTURAL CHARACTERISTICS OF PASTEURELLA TULARENSE

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There has been relatively little work published on the cultural characteristics of *Pasteurella tularense*. Previous articles have been concerned mainly with the description of media favorable to its growth. McCoy and Chapin (1912) first isolated the organism on coagulated egg medium and Francis in 1922 reported that the addition of cystine to blood or serum agar stimulated the growth of the organism. Francis,[2] using glucose, mannose, levulose and glycerol which were added to cystine agar slants containing brom-thymol-blue as an indicator found that the three or four strains tested by him fermented these carbohydrates. Shaw (1930) however, reports that two human strains isolated by him show no fermentation of glucose.

In view of these somewhat meager reports and in connection with a study of the fermentation of the *Pasteurella* group as a whole the following studies were undertaken.

The organisms used were collected from various sources.[3] The origin of the different strains is as follows: isolated from human cases, seven; rabbits, four; ground squirrels, one; wood ticks, three; field mice, one; fox, two; grouse, one; muskrat, one; quail, one; twenty-one in all.

[1] Part of the funds used to carry on these experiments were kindly granted by the Research Committee of the Graduate School of the University.

[2] Personal communication.

[3] My thanks are extended to Dr. R. G. Green of the University of Minnesota and to Dr. Edward R. Francis of the National Institute of Health for their kindness in supplying a number of these strains.

FERMENTATION REACTIONS

The basic medium was meat-extract broth, 2 per cent peptone and 1.5 per cent agar to which cystine hydrochloride was added to make 0.01 per cent. The reaction was adjusted to pH 7.4, and enough phenol-red indicator was added to give a distinct salmon pink shade. The carbohydrates were added to make a final concentration of 1 per cent. The medium was then sterilized in the autoclave and before tubing 5 per cent of horse serum was added, and the tubes slanted. Arabinose, xylose, rhamnose; glucose, galactose, mannose, levulose; lactose, maltose, sucrose; melizitose, raffinose; dextrin; mannitol, glycerol, sorbitol, dulcitol; salicin were the carbohydrates used. The media were inoculated with the 21 strains of *Pasteurella tularense*, incubated at 37°C. and observed daily. In all cases good growth occurred on the slant. Glucose and glycerol were fermented by all the strains, mannose was fermented by all but two; levulose by all but five; maltose by all but one. The other carbohydrates were not fermented. Francis who used three or four strains of *Pasteurella tularense* found that all of them fermented glucose, mannose, levulose and glycerol. He apparently did not use maltose. It is apparent that our results agree closely with those of Francis except for the few strains which were negative in mannose and levulose. These negative strains were carefully checked but always failed to give fermentation although they grew well. It will be noted that all of our strains fermented glucose as did those of Francis whereas Shaw reports that his two human strains did not.

An interesting aspect of these fermentation tests was the increase in alkalinity in all media in which the carbohydrate was not fermented. The color of the phenol red in these tubes showed a deep pink after the fourth day and reached a reaction of approximately pH 8.0 to 8.4 after the seventh day. The tubes showing fermentation reached a pH of 6.4 to 6.6 on the seventh to ninth day and then slowly decreased in acidity until at the seventeenth to the twenty-third day they also showed a reaction of pH 8.0 to 8.4.

Bond and Downs in a study of the *Pasteurella* group used the

above carbohydrates but were not able to group the organisms according to their fermentation reaction nor to find that the fermentation reactions of *P. tularense* showed any close resemblance to those of the other members of the group.

HYDROGEN SULPHIDE PRODUCTION BY PASTEURELLA TULARENSE

Because of the stimulative effect of cystine on the growth of *P. tularense* it was thought probable that it might utilize this sulphur compound with the production of hydrogen sulphide. For this study a basic slant medium of semi-solid, glucose, 2 per cent Bacto-peptone, meat-extract agar containing cystine or cystine hydrochloride was used. Ferrous sulphate was added as an indicator in some series, in others a strip of moistened lead acetate paper was introduced into the tube after seven days incubation and the tubes tightly stoppered. Sixteen strains were tested, all of which gave positive tests for hydrogen sulphide. A series of tests was then made to find out if the cystine was the source of the hydrogen sulphide. For these tests the basic medium was glucose meat-extract agar to which was added the following:

1. Basic medium plus 5 per cent rabbit blood
2. Basic medium plus 2 per cent peptone
3. Basic medium plus blood and peptone
4. Basic medium plus sodium thiosulphate
5. Basic medium plus sodium thiosulphate plus blood
6. Basic medium plus sodium thiosulphate plus peptone
7. Basic medium plus sodium thiosulphate plus peptone and blood

There was no hydrogen sulphide production on any of these media although there was fairly good growth. These results would suggest that cystine was the source of the hydrogen sulphide. Although there is cystine present both in the peptone and blood the use of additional cystine stimulates growth and the added cystine is apparently largely broken down into H_2S. Almy and James (1926) using *Proteus vulgaris* inoculated into 100 cc. of a peptone medium to which 0.01 gram of cystine was added recovered the theoretical amount of H_2S to be expected from the breakdown of 0.01 gram of cystine. It is a common experience to be able to demonstrate H_2S production by *Escher-*

ichia coli in a medium to which cystine is added, whereas in the ordinary peptone medium it is not detectable with lead acetate paper or a ferrous sulphate indicator. Almy and James (1926), Tanner (1917), Sasaki and Otsuka (1912) have all shown cystine to be readily available as a source of H_2S while Burrows (1934) Scheff and Scheff (1934) and others have shown that cystine has a stimulative effect on growth. Cultures of *P. tularense* in a cystine medium evidently show both of these effects. It is evident that *P. tularense* can not utilize sodium thiosulphate as a source of hydrogen sulphide as many organisms do. Treece[4] has shown this substance to be an excellent source of hydrogen sulphide in the *coli* and *Salmonella* groups. Tarr (1933) has shown that whereas *Proteus vulgaris* produces hydrogen sulphide from sodium thiosulphate, *Erythrobacillus prodigiosus* can not form it from this source. Both organisms however produce hydrogen sulphide from cystine.

GROWTH ON MEDIA CONTAINING VARIOUS CHEMICAL COMPOUNDS

Because of the marked stimulative effect of cystine on the growth of *P. tularense* it was decided to try out a series of sulphur compounds to see if any of them could be used as a substitute for cystine. Francis (1923) reports on a series of amino acids, among them cystine and tryptophane, and also on inorganic sulphur compounds which were added to agar used for the inoculation of *P. tularense*. He found that only cystine or cystine hydrochloride supported growth. The following sulphur compounds failed to support growth: sodium sulphite, sodium hydrogen sulphite, sodium thiosulphate, ammonium sulphate, magnesium sulphate, potassium sulphate, resublimed and precipitated sulphur. The possible inhibitive effect of these substances was not tested for.

In our series of tests the same compounds were used and in addition; urea, thio-urea, ferric ammonium citrate, ferrous sulphate and sodium nitrate and nitrite. These were added to make a final concentration of 0.1 per cent to each of two series of tubes. In one series cystine was added. Five per cent horse serum and

[4] E. L. Treece, personal communication.

1 per cent glucose was added to all of the media. There was slight growth on all of the media containing no cystine but there was nothing to indicate that any of the substances used had a stimulative effect similar to that of cystine. The tubes containing the sulphur compounds and other salts plus cystine showed the same abundant growth seen in the control tubes of glucose, cystine serum agar. These compounds therefore showed no inhibitive effect on the growth of *P. tularense*.

GROWTH ON SYNTHETIC AND SEMISYNTHETIC MEDIA

A study of the cultural characteristics of *P. tularense* is rather difficult because of its growth requirements. It will not grow on liquid media and it seems to require blood or serum, in addition to cystine to give abundant growth. Scott and Brandly (1933) have reported the use of a synthetic medium which seems to give abundant growth with a large number of different species of bacteria. Accordingly it was considered desirable to see if *P. tularense* would grow on such a relatively simple medium. No growth was obtained on this medium. However, when 5 per cent horse serum or rabbit blood was added a fair but not an abundant growth occurred.

The further addition of glucose to this medium had a marked stimulative effect on growth. The synthetic medium with the addition of cystine but without serum or blood showed slight but unmistakable growth. This medium containing cystine and blood or serum seemed to be as favorable for growth as the usual meat extract agar. However, it is in this case scarcely more simple than the usual medium.

SUMMARY AND CONCLUSIONS

1. Twenty-one strains of *Pasteurella tularense* were found to ferment glucose and glycerol. Most of the strains also fermented mannose, levulose and maltose. All strains failed to ferment galactose, lactose, sucrose; raffinose, melizitose, arabinose, rhamnose, xylose; mannitol, dulcitol, sorbitol; salicin.

2. *P. tularense* produces an alkaline reaction in media not containing a utilizable carbohydrate; but, in the presence

of a utilizable carbohydrate it produces an initial acidity followed by an alkaline reversion.

3. *P. tularense* produces hydrogen sulphide in media containing cystine.

4. *P. tularense* is apparently unable to produce hydrogen sulphide in detectable amounts from media containing peptone and blood or serum.

5. *P. tularense* does not produce H_2S in detectable amounts from sodium thiosulphate.

6. The addition of a number of sulphur-containing compounds failed to stimulate the growth of *P. tularense* but in the presence of these compounds there was no inhibition of the usual stimulative effect of cystine.

7. Scott and Brandly's synthetic medium with the addition of blood or serum supports the growth of *P. tularense*.

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THE AMYLASE OF CLOSTRIDIUM ACETOBUTYLICUM

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Glucose, isolated and identified as the crystalline osazone, was shown by Speakman (1920) to be a product of the activity of *Clostridium acetobutylicum* in a maize medium to which toluene was added a few hours after inoculation. Robinson (1922) observed that during the progress of an active maize fermentation reducing sugar accumulated in the medium, and that, later, it almost completely disappeared through fermentation. Obviously an active amylase was elaborated by the organism. The present paper contains an account of some experiments on the preparation and behaviour of this enzyme. In view of the importance now attached to the initial constant velocity of reaction in enzyme studies the investigations have been based on methods which permit an examination of the progress of the reaction during the first few minutes of contact between enzyme and substrate.

MATERIALS AND METHODS

Enzyme preparation

Attempts to demonstrate the presence of amylase in cells separated from the culture medium were unsuccessful. Several preparations were employed: washed organisms, air-dried cells ground to a fine powder, autolysed organisms (wet and dry), acetone-treated cells, cells subjected to grinding with sand, and, finally, cells alternately frozen and thawed several times. However, active precipitates were obtained by adding alcohol or acetone in suitable amounts to the glucose-peptone medium from which the organisms, after forty hours' incubation at 37°, were removed by means of the Sharples centrifuge. Unlike the amy-

lase, the phosphatase formed by this organism is not secreted into the external medium but can be readily demonstrated in dried cells (Pett and Wynne, 1933).

Two types of enzyme preparation have been used in this work:

Type 1. Sterile 5 per cent maize mash containing 0.02 M sodium acetate was inoculated and allowed to ferment until the residual maize collected in the characteristic "head." A clear solution was obtained by passage first through cheesecloth, then through filter paper and finally through the supercentrifuge. The clear liquid was concentrated at 30° to one-twentieth its volume and the concentrate was centrifuged for thirty minutes at 1800 r.p.m. Subsequent Seitz filtration gave a very clear preparation. Solutions prepared in this manner usually contained about 100 "liquefaction units" per cubic centimeter; this unit represents the amount of amylase necessary to reduce by 20 per cent the viscosity of 4.1 cc. of starch-enzyme solution containing 2.5 per cent Lintner soluble starch (Merck), in one hour, at 37° and pH 4.95.

Amylase preparations of this type are, of course, quite impure and contain considerable amounts of reducing substance, some of which is not fermented by yeast. The reducing material can be largely removed by ordinary or electrical dialysis, but attempts to remove it completely result in an irreversible inactivation of the enzyme.

Type 2. The first preparation was electrically dialysed against running distilled water until it lost about one-third of its original activity. The insoluble material in the enzyme solution was removed and the amylase was precipitated from the clear solution by adding enough alcohol to give a final concentration of 85 per cent. The precipitate was quickly separated by centrifuging and was dried *in vacuo* over sulphuric acid. In this manner preparations have been made containing more than 5000 liquefaction units per gram. The preparations were almost free from reducing substances but they were much less stable than those of the first type. In view of this instability, further purification was not considered advisable for the purposes of the present investigation.

Buffer. M/50 or M/100 acetic acid-sodium acetate.

Starch substrate

For liquefaction experiments, fresh 3.5 per cent aqueous solutions of Lintner soluble starch (Merck) were prepared each day. For saccharification studies Lintner soluble starch was pre-treated with 7 per cent HCl for twelve hours at 37° with frequent shaking. The material was washed several times with distilled water until just alkaline to methyl orange. The starch was then dried and preserved in a desiccator; it possessed practically no reducing power and the viscosity of a 3 per cent solution was only slightly higher than that of water.

Measurement of amylase activity

Liquefaction method. Three cubic centimeters of a 3.5 per cent solution of Lintner starch, 0.1 cc. of buffer solution or of distilled water and 1 cc. of amylase solution were introduced into an Ostwald viscosimeter and the decrease in the viscosity of the mixture was followed while the instrument was immersed in a glass-walled thermostat. The temperature was 37°±0.2 and all times of flow were measured by stop watch to the nearest tenth of a second. The times of flow (seconds) recorded after successive intervals were plotted (as ordinate) against digestion time (minutes); the initial time of flow of the digestion mixture before any hydrolysis occurred was obtained by extrapolation to zero time. The initial viscosity was given a value "100 per cent viscosity"; subsequent values were calculated as per cents of this initial value on the basis of the relative times of flow. From the chart correlating relative viscosity and time of digestion, the time in minutes (x) required for the reduction of the viscosity to 80 per cent of the initial value was read, and the quotient obtained by dividing 60 by (x) was taken to represent (when 1 cc. of enzyme solution was used) the number of amylase units per cubic centimeter of enzyme solution.

Saccharogenic method. The amount of reducing substance present in the enzyme digests was determined by the Hagedorn-Jensen method as modified by Hanes (1929) and by Hulme and Narain (1931). Whenever it was necessary to precipitate the proteins from the sample, this was done by means of zinc hydroxide in the manner described by the original authors (Hagedorn

and Jensen, 1923). Inactivation of the enzyme was accomplished by adding each sample directly to the Hagedorn-Jensen solutions. For each digest a progress curve was drawn, correlating time and the accumulation of reducing sugar, and the initial velocity of hydrolysis was determined by drawing a tangent to the curve at a point near the origin. With the higher concentrations of substrate the progress curves were frequently linear over a period of eight to ten minutes.

EXPERIMENTAL RESULTS

Influence of pH on the activity of the enzyme

This was investigated by the liquefaction and saccharogenic methods with amylase preparations of the first and second types respectively. The pH of the reaction mixtures was adjusted by adding HCl or NaOH; buffer was not used since no significant pH change occurred during the relatively short digestion periods. The temperature was 37°. Both methods yielded essentially the same result. With the liquefaction method the optimum range was pH 4.8 to 5.0, the curve dropping symmetrically to zero activity at pH 1.5 and pH 7.5. With the saccharogenic method the optimum range was pH 4.85 to 5.05, the curve dropping to zero activity at pH 2 and to one-fourth of the optimum activity at pH 7.3.

Influence of sodium acetate on the activity of the enzyme

Previous experiments had shown that the presence of suitable amounts of sodium acetate in culture media containing maize mash or starch-peptone was associated with an increase in the rate of fermentation. At the optimum concentration of 0.015 M to 0.03 M sodium acetate an acceleration of 25 per cent was frequently observed. The rate of fermentation of glucose peptone was, however, not accelerated by this salt. In fact, amounts of the salt which accelerated the fermentation of starch media were sufficient to retard very appreciably the rate of fermentation of glucose peptone. It appeared possible that the effect of sodium acetate in starch-containing media was associated with an activation of the amylase system. This point was investigated by the

liquefaction and saccharogenic methods and with different amylase preparations. In no case did sodium acetate accelerate the rate of starch hydrolysis in an enzyme digest. In two of the several experiments, dialysed starch and re-dialysed enzyme (Type 2) were used. The enzyme preparation was made by suspending in distilled water sufficient amylase preparation, obtained from a culture containing no added sodium acetate, to give a solution containing 70 units per cubic centimeter. This solution was then electrically dialysed against distilled water for twenty-five hours during which time its potency was reduced to 11 units per cubic centimeter. Equal amounts of the enzyme so'ution were allowed to act upon the dialysed starch (1 per cent) at 37° and pH 4.95 in the presence of sodium acetate in concentrations ranging from 0.002 M to 0.03 M. Buffer solution was not added: no significant pH change occurred in any digest in the short incubation period adopted. The amounts of reducing sugar formed in nine minutes were determined: in every case, added sodium acetate caused a diminution in reducing sugar, the retardation of enzyme activity increasing with increase of sodium acetate concentration.

Though this salt apparently exercises this effect on the enzyme system *in vitro* its presence in the culture medium augments the amount of amylase in the medium at the end of fermentation. This was shown by estimating the relative amylase content of the filtered solutions obtained from seventy-hour maize cultures to which varying amounts of sodium acetate had been added before inoculation. The estimations were made in two ways: (1) by determining the time required for a given quantity of the clear liquid from each flask to hydrolyse a standard amount of starch to the point where it no longer gave a color with iodine; (2) by determining the number of liquefaction units per cubic centimeter. The concentration of sodium acetate in the various cultures varied from 0.01 M to 0.06 M. The presence of sodium acetate in every case caused an increase in the total amylase in the medium at the end of the fermentation. Using the iodine method the optimum concentration of the salt was found to be 0.01 M to 0.02 M; using the liquefaction method the optimum con-

centration was 0.035 M to 0.045 M. In previous experiments it was observed that it was in this range of concentration that sodium acetate was most effectual in accelerating the rate of fermentation of maize and starch-peptone by the organism. Other effects of sodium acetate on the metabolism of the organism will be discussed in another paper.

Influence of phosphate and chloride on the activity of the enzyme

Added phosphate and chloride in concentrations ranging from 0.01 M to 0.04 M and from 0.009 M to 0.037 M, respectively, caused retardation of the rate of hydrolysis at pH 4.95.

Relation between substrate concentration and reaction velocity

In studying this relationship a Type 1 amylase preparation was used. The substrates were made up from a solution of soluble starch prepared by HCl treatment in the manner previously described. All reactions were carried out at 37.5° and pH 4.95 in the presence of acetate buffer. The progress of each reaction was followed for about twelve minutes by determining the reducing sugar at one- or two-minute intervals; progress curves were plotted and initial velocities were calculated. The values of K_s and V_{max} in the well known mass-law expression of Michaelis and Menten (1913)

$$v = \frac{V_{max}\, S}{S + K_s}$$

were derived by substituting experimental values of v (observed initial velocity) and S (substrate concentration) in two equations normal with respect to $1/V_{max}$ and K_s/V_{max}, respectively, and solving in the ordinary manner. K_s was found to be 0.050 per cent and V_{max} 1.79. Substituting these values in the original equation, the latter now becomes

$$v = \frac{1.79\, S}{S + 0.050}$$

From this equation the theoretical velocities corresponding to various concentrations of substrate were calculated. Along with

the observed values these are recorded in table 1. The close agreement between observed and calculated velocities indicates that the enzyme behaves in conformity with the theory of Michaelis and Menten which states that enzyme and substrate react according to the mass law to form a compound whose concentration determines the speed of the observed reaction. Hanes (1932)

TABLE 1

Relation between initial velocity of hydrolysis and substrate concentration

STARCH CONCENTRATION [S]	OBSERVED INITIAL VELOCITY	CALCULATED VELOCITY $v = \frac{1.79\,[S]}{[S] + 0.050}$
per cent		
0.02	0.50	0.51
0.05	0.93	0.89
0.08	1.13	1.10
0.15	1.33	1.34
0.30	1.49	1.53
0.50	1.61	1.63
1.00	1.72	1.70

TABLE 2

Effect of temperature on the initial velocity of hydrolysis

TEMPERATURE RANGE	0.1 PER CENT STARCH		1.0 PER CENT STARCH	
	Q_{10}	E	Q_{10}	E
degrees		*calories*		*calories*
10–20	1.42	5744	2.14	12510
20–30	1.45	6572	1.74	9329
30–40	1.44	6779	1.35	5671
35–45	1.46	7381	1.25	4347
40–50			1.19	3554

showed that the barley amylase-starch system also behaves in a manner predictable by the Michaelis theory. His values for K_s were 0.077 and 0.079 per cent in two experiments.

The relation between enzyme concentration and reaction velocity was examined with the aid of the liquefaction and saccharogenic methods. Over a considerable range of enzyme concentration the realtionship was linear in both experiments.

Influence of temperature on the rate of hydrolysis

The hydrolysis of 0.1 and 1.0 per cent starch by Type 1 amylase was followed at pH 4.95 and at various temperatures. Progress curves were drawn and from these the initial velocities were determined. The latter values were plotted against temperature and from the curves the temperature coefficients (Q_{10}) were calculated.

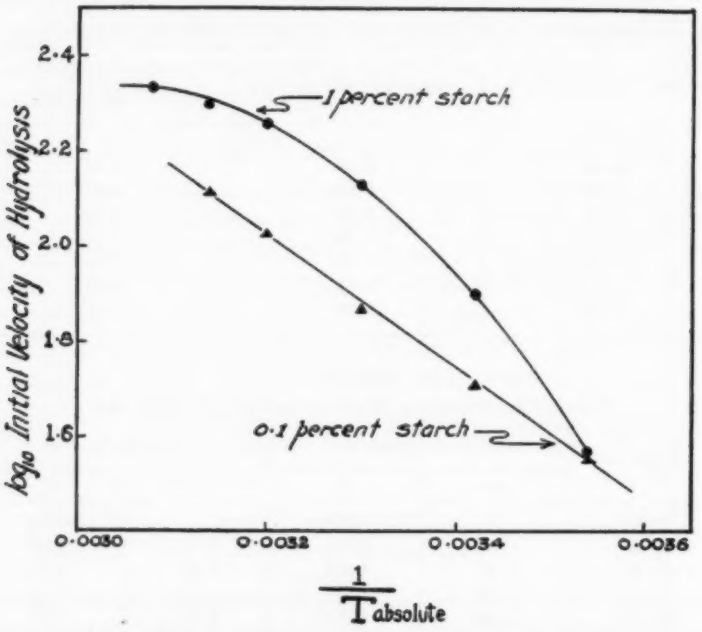

FIG. 1. INITIAL VELOCITY OF HYDROLYSIS AS A FUNCTION OF TEMPERATURE
(Johnston and Wynne: Amylase of *Cl. acetobutylicum*)

In the case of 0.1 per cent starch Q_{10} varied only between 1.42 and 1.46 over the temperature range 10 to 46°; with 1 per cent starch Q_{10} varied from 2.14 to 1.19 over the same range (table 2).

Attempts have frequently been made to extend the consideration of temperature effects to include the question of the so-called energy of activation and its significance in enzyme reactions.

Although, as Hinshelwood (1933) states, the Arrhenius equation

$$\frac{d \log k}{dT} = \frac{E}{RT^2}$$

applies with accuracy in many gaseous reactions and the term E in this equation represents the critical thermal increment neces-

TABLE 3

Inactivation of the enzyme by heat

TEMPERATURE	TIME	INACTIVATED ENZYME AS PER CENT OF ORIGINAL	K_{usl}	INITIAL RATE OF INACTIVATION	E^*
	hours			*per cent per hour*	*calories*
50.5°	0	0			
	1.17	49.4	0.583		
	2.17	71.4	0.570	55	38,970 (45.5–50.5°)
	4.25	90.6	0.535		
	5.42	93.1	0.295		
45.5°	0	0			
	2.0	38.1	0.239		
	4.5	65.0	0.225		
	8.25	87.7	0.280	21.25	31,900 (37.5–50.5°)
	18.5	93.5	0.060		
	39.0	98.6	0.074		
37.5°	0	0			
	3.5	24.1	0.0789		
	6.5	42.3	0.0794		
	9.5	51.0	0.0663	6.9	27,660 (37.5–45.5°)
	11.5	57.3	0.0685		
	23.0	60.8	0.0073		
30.5°	0	0			
	4.5	21.6	0.0540	6.4†	
	18.0	38.0	0.0175		14,199 (30.5–37.5°)
	27.5	34.7			
	41.5	36.5			

* Temperature range in parentheses.

† Approximate.

sary for the activation of the reacting gaseous molecules, nevertheless attempts to transfer such considerations to reactions catalysed by enzymes and to physiological systems in general are

justifiable only on quite empirical grounds and have not yet led to any clear concept of activation in biochemical reactions. Therefore the results of the present experiments are presented for purposes of record only: we are not yet prepared to suggest any adequate interpretation of their significance.

Figure 1 indicates that, in the case of the hydrolysis of 0.1 per cent starch, E is fairly constant over a considerable temperature range, being represented by the slope of the straight line. The continuous change in the slope of the other line (1 per cent starch) might be interpreted as indicating a composite reaction made up of several concurrent reactions each characterised by a different value of E. The values of E calculated from the Arrhenius equation, together with the temperature coefficients, for several temperature ranges are recorded in table 2. For 0.1 per cent starch, E is fairly constant, but for 1 per cent starch the value of E drops almost linearly with rise of temperature between the limits 15° and 40°.

Inactivation by heat

The rate of inactivation of a Type 1 amylase preparation at pH 4.95 and at the temperatures 30.5°, 37.5°, 45.5°, and 50.5° was followed by periodic determination of the number of residual liquefaction units in a standard volume of enzyme solution incubated for several hours. The unimolecular constants were calculated and were found, in all cases, to fall off sharply in the latter stages although the initial course of the inactivation process conformed reasonably well with the unimolecular expression. The data are recorded in table 3.

In applying the data to the Arrhenius equation, use was made, not of the calculated K_{uni} values, but rather of the initial rates of inactivation expressed as per cent inactivation per hour and derived from curves obtained by plotting per cent inactivation against time. The calculated values of E (table 3), when plotted against temperature, are seen to rise linearly with temperature. Pace (1931) has obtained for proteolytic enzymes figures of a similar order of magnitude and regards them as representing the critical thermal increments necessary for inactivation.

SUMMARY

1. The rate of hydrolysis of starch by preparations containing the amylase of *Clostridium acetobutylicum* was studied in relation to pH, salt effects, substrate and enzyme concentration, temperature.

2. A preliminary investigation of the inactivation of the enzyme by heat was made.

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THE ACTION OF RADIATION IN THE EXTREME ULTRA-VIOLET ON BACILLUS SUBTILIS SPORES

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The germicidal action of parts of the extreme ultra-violet spectrum on living organisms has been discussed by Bovie (1916). The radiation used by Bovie, from a hydrogen discharge tube with a fluorite window, did not permit him to examine the effect of radiant energy below 1250 Å. Since this time, the work of Hopfield (1922), which was later extended by Ladenberg, Van Vorrhis, and Boyce (1932), has shown that though the air is generally considered opaque from the line 1850 Å, a definite transmission band exists between 1100 Å and 1300 Å. Also, some crystals of lithium fluoride have been shown to be transparent to radiation down to 1100 Å. These discoveries permit the use of very simple techniques for the investigation of the effects of the extreme ultra-violet.

In this work, we used a condenser of 0.016 microfarads capacity in parallel with a 15,000 volt sign-lighting transformer and a carbon electrode spark gap. Strong lines of oxygen and nitrogen are present in the radiant energy from such a spark and the region within 4 to 5 cm. of the spark can be shown by the method of Lyman (1935) to be rich in the extreme ultra-violet radiations.

A five-day old extract agar slant culture of *Bacillus subtilis* was suspended in 0.9 per cent NaCl solution and heated at 70°C. for one hour. This suspension was then swabbed uniformly onto the surface of 5 ml. of medium in a 4.5 cm. petri dish. The medium contained 0.15 gm. beef extract, 0.5 gm. NaCl and 2.0 gm.

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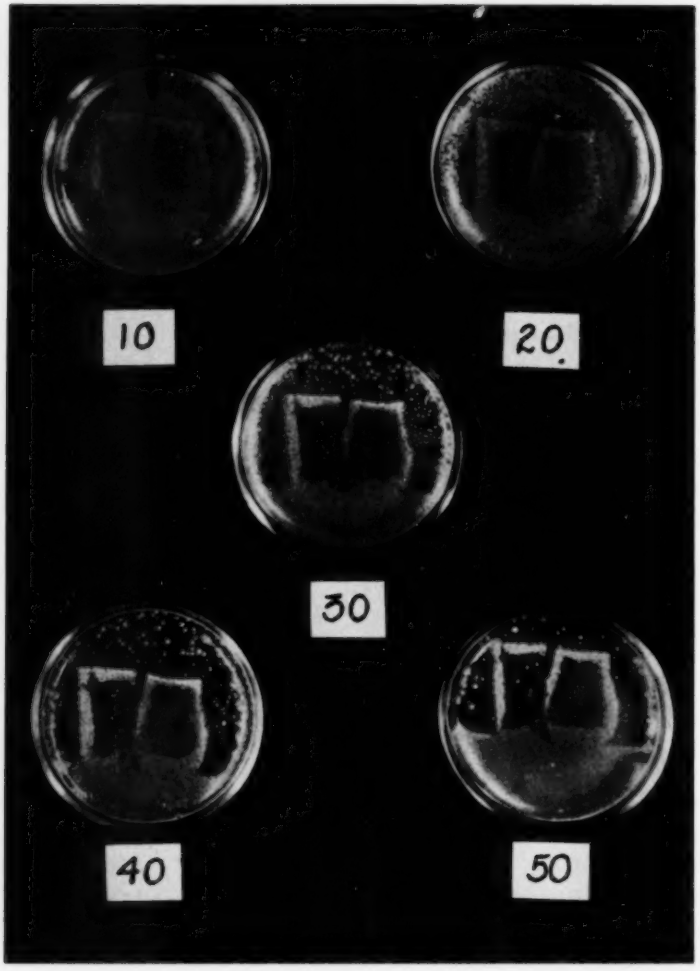

FIG. 1. SHOWING THE EFFECT OF THE EXTREME ULTRA-VIOLET ON THE GROWTH OF BACILLUS SUBTILIS SPORES

Time of irradiation in seconds. Left crystal transmits down to 1100Å; right crystal down to 1400Å.

agar per 100 ml. After inoculation of the dishes, they were held open at 37.5°C. for 2 hours to dry the inoculated surface. This was necessary in order to remove the water which would have acted as a shield for the organisms and also to prevent the spreading of the viable organisms over the surface of the medium by capillarity.

Immediately after drying, the uncovered plates were placed so that the spark gap was directly over the center of the plate at 2.5 cm. from the surface of the medium. Over the top of the plate were then placed two small crystals of lithium fluoride[3] fastened at the edge of a microscope slide. One of these crystals transmitted radiation down to 1100 Å; the other to 1400 Å; the microscope slide to approximately 3500 Å. Five plates, irradiated in this manner for 10, 20, 30, 40, and 50 seconds respectively and subsequently incubated at 30°C. for 48 hours, are shown in figure 1. In each case the crystal on the left is the one which transmits down to 1100 Å. The bottom part of the dish is that part covered by the microscope slide. The oblique edges of the crystals cast shadows and the spores in these shadows remained uninjured and subsequently grew, showing the outline of the crystal.

The very sparse growth under the left crystal as compared to the almost confluent growth under the right crystal plainly shows the effect of the extreme ultra-violet in the region between 1100Å and 1400 Å. So far as we are aware, this is the first specific proof of the antiseptic or germicidal action of this region of the ultra-violet. No attempt has been made here to distinguish between the effect of the radiation exerted directly on the organisms and any secondary effect resulting from changes in the culture medium. The latter, however, is thought to be negligible and no inhibition of growth could be observed when the medium alone was irradiated for as long as 15 minutes previous to inoculation.

The authors wish to acknowledge their indebtedness to Prof. T. Lyman and Dr. E. G. Schneider for the helpful suggestions offered during this investigation.

[3] Dr. E. G. Schneider, Research Laboratory of Physics, Harvard University, kindly provided us with these crystals, the transmission of which he had measured.

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THE INHIBITION OF GROWTH OF BACILLUS SUBTILIS BY ULTRA-VIOLET IRRADIATED CARBOHYDRATES

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While investigating the killing of the spores of the anthrax bacillus by sunlight, Roux (1887) came to the conclusion that germination of the spores could be inhibited by materials which were formed as a result of the oxidation of the carbohydrate fraction of the medium. Most subsequent work of this type has been concerned with the action of ultra-violet radiation directly on the organisms and has been reviewed by Laurens (1933). Coblentz and Fulton (1924), Woodrow, Bailey and Fulmer (1927) and Proks (1933) showed that the ability of a medium containing carbohydrates to support the growth of microörganisms was diminished if the medium was irradiated with ultra-violet energy previous to inoculation.

One of the authors (Blank and Kersten (1935)) showed that a modified extract agar could be so altered by the action of soft x-rays that it would no longer support the growth of *Bacillus subtilis*. This inhibition results from the formation of a toxic material from the agar—the major carbohydrate of the medium. Experimental proof is presented here to show that ultra-violet radiation produces similar effects. It is further shown that the growth of *Bacillus subtilis* is prevented by adding to the medium an ultra-violet irradiated solution of any one of a number of different carbohydrates.

Our source of ultra-violet energy was a quartz mercury discharge tube, 8 mm. in diameter and operated by a 5000-volt sign-

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lighting transformer. Approximately 90 per cent of the radiation from this tube is in the line 2537 Å. The materials being irradiated were held in 4.5 cm. petri dishes at 5.0 cm. from the tube at which point the incident energy was 47 ergs per square millimeter per second.

The bacteriological techniques, i.e., the preparation of the medium and inoculum, method of inoculation, incubation, etc., were identical with those used for the x-ray work (Blank and Kersten (1935)).

Ultra-violet irradiation of 10 ml. of the entire culture medium (0.15 per cent beef extract, 0.5 per cent NaCl and 2 per cent agar) under the conditions specified above for four hours so alters the medium that it will no longer support the growth of *Bacillus subtilis*. As the volume of medium is decreased, the time of irradiation necessary to bring about complete inhibition is correspondingly decreased.

Incorporation of irradiated beef extract, sodium chloride, water, or water solutions of the extract or the salt into culture media does not produce media which completely inhibit the growth of *Bacillus subtilis*. When media are prepared from irradiated powdered agar or agar-water gels, however, these media do not support a growth of *Bacillus subtilis*. Complete inhibition results when a 2.5 ml. quantity of a 4 per cent agar gel is irradiated for three hours and subsequently made into 5 ml. of medium. Powdered dry agar must be irradiated for a longer time to bring about complete inhibition.

When irradiated 2 per cent agar gels are soaked in distilled water, the gel removed, the soak waters evaporated to dryness on a steam bath, and the resulting solids incorporated into a culture medium, this medium no longer supports the growth of *Bacillus subtilis*. When an irradiated 2 per cent agar gel is evaporated to dryness under vacuum, the medium prepared from the residue inhibits the growth of *Bacillus subtilis*. Ability to support growth is not restored to an irradiated medium by autoclaving it at 15 lbs. pressure for fifteen minutes.

It is thus seen that the effects of irradiating bacterial culture media with ultra-violet energy at 2537 Å exactly parallel the

effects of 1.54 Å radiation as previously reported (Blank and Kersten (1935)). A thermostable, non-volatile material that will diffuse through and from the gel is formed by the action of the radiant energy on agar or agar-water gels. This material prevents the germination of the spores of *Bacillus subtilis* and the subsequent formation of macroscopically visible colonies. It is not yet known whether one and the same toxic material is produced by both types of radiant energy.

TABLE 1

Irradiation time which will produce in a 1 per cent solution of the carbohydrate sufficient toxic material so that no colonies of B. subtilis develop on the subsequently prepared medium after seventy-two hours (incubation at 30°C.)

CARBOHYDRATE	TIME	CARBOHYDRATE	TIME
	hours		*hours*
Pentoses:		Trisaccharides:	
l-Xylose	1.5 to 2.0	Raffinose	1.5 to 2.0
d-Arabinose	1.5 to 2.0		
l-Arabinose	1.5 to 2.0		
Hexoses:		Polysaccharides:	
d-Levulose	0.5 to 1.0	Inulin	1.0 to 1.5
d-Glucose	1.0 to 1.5	Soluble starch	1.5 to 2.0
d-Mannose	1.5 to 2.0	Dextrin	2.0 to 2.5
d-Galactose	1.5 to 2.0	Corn starch	2.5 to 3.0
i-Inositol	1.5 to 2.0	Glycogen	5.0 and up
Disaccharides:		Derivatives:	
Saccharose	1.0 to 1.5	Amygdalin	0.0 to 0.5
Lactose	1.5 to 2.0	Salicin	0.5 to 1.0
Cellobiose	1.5 to 2.0	Rhamnose	2.5 to 3.0
Maltose	2.0 to 2.5		
Trehalose	2.5 to 3.0		
Melibiose	2.5 to 3.0		

The discovery that irradiation of culture media inhibits the growth of *Bacillus subtilis* because of changes in the agar fraction of the medium has led to the investigation of the action of ultraviolet radiation on other carbohydrates. Two and one-half-milliliter quantities of solutions of carbohydrates (1 gm. per 100 ml. of water) were irradiated for varying periods of time. Following irradiation, the water lost by evaporation was restored with sterile

distilled water; 2.5 ml. of a hot, sterile solution containing 4.0 gm. agar, 0.3 gm. of beef extract and 1.0 gm. NaCl per 100 ml. were aseptically added, well mixed, allowed to gel, and inoculated with a suspension of *Bacillus subtilis* spores using a sterile swab. Table 1 shows the approximate time of irradiation which will produce in each of the carbohydrate solutions a sufficient amount of toxic material to prevent the formation of any macroscopically visible colonies on the subsequently prepared medium after seventy-two hours incubation at 30°C. All control media showed normal confluent growth in less than twenty-four hours incubation. Uncontrollable variation in inoculum and possibly other unrecognized factors make it difficult to determine the exact time of irradiation required to produce complete inhibition.

It should be observed that in each of three classes of carbohydrates—hexoses, disaccharides, and polysaccharides—the irradiation of the ketose, or the carbohydrate which hydrolyzes to produce a ketose, brings about complete inhibition with a shorter period of irradiation than is required for any aldose in that class. This parallels the work of Berthelot and Gaudechon (1912) who showed that for equal periods of irradiation, greater chemical changes occurred in the ketoses than in the aldoses.

It should also be noted that complete inhibition was brought about by irradiated glycogen only after it had been given at least twice the dosage used for any other carbohydrate. The term—"5 and up"—in table 1 indicates that samples of glycogen obtained from different manufacturers required different dosages, five hours being the minimum. Some showed no ability to inhibit growth even after seven hours irradiation. The reason why glycogen should require so much larger dosage than other compounds of similar structure, dextrin for example, is not yet apparent. The purity of the glycogen samples was not determined and the rôle played by any impurity is not known. The presence of an alkali, for instance, may have a marked effect. The entire question of the influence of the hydrogen ion concentration of the carbohydrate solution during irradiation must yet be investigated.

CONCLUSIONS

1. Ultra-violet radiation (2537 Å) so alters agar or agar-water gels that when culture media are subsequently made from them, they will not support the growth of *Bacillus subtilis*. This results from the formation of a non-volatile, thermostable material, capable of diffusing through and from the agar gel, which prevents the growth of the organism.

2. *Bacillus subtilis* can be similarly inhibited by the addition to the culture medium of an irradiated solution of any one of 20 different carbohydrates and 3 carbohydrate derivatives.

The authors wish to acknowledge their indebtedness to Professors William H. Weston, Jr. and W. J. Crozier for the helpful suggestions offered during this investigation.

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STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA[1]

VI. THE DIPHTHERIA BACILLUS

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Earlier work (Mueller, Klise, Porter and Grabiel, 1933) has shown that for the growth of a particular strain of diphtheria bacillus which has been investigated—a moderately strong toxin producer, isolated some years ago by Dr. Ho Yu in this laboratory—a combination of meat extract, amino acids and inorganic salts was required for successful growth. The amino acid requirements, in the presence of a crude tissue extract (Liebig's extract of meat) have now been fairly well defined for this strain (Mueller, 1935a). In the preliminary work a certain amount of information was obtained as to chemical methods for the separation of the constituents of the meat extract, using a crude acid hydrolysate of edestin, in place of pure amino acids, as a control. It was stated, in reporting this work, (Mueller, Klise, Porter and Grabill, 1933) that either the greater part of the substance in Liebig's extract, which seemed essential for growth, was lost during the fractionation, or else that additional factors—concerning the nature of which there was no clue—would be found to be involved. Since it is now possible to substitute pure, and to a certain extent, synthetic amino acids for the crude edestin or other protein hydrolysate, we are in a better position to re-

[1] The writer is indebted to Professor N. B. Dreyer of the department of Pharmacology and Professor R. P. Smith of the Department of Pathology, of Dalhousie University, Halifax, for their courtesy in extending him the hospitality of their laboratories, where a part of this work was carried out during the summer of 1935.

investigate the tissue extract fraction. Moreover, the quantitative method of estimation of growth now being used (Mueller, 1935b) provides a much more accurate means of following through a series of experiments of this kind. This paper will therefore deal with a further study of the action of the extractives.

METHODS

The same methods used in the experiments to which reference has already been made, are used throughout the present work. The various control substances are added in the amounts shown in table 1 together with stated quantities of the variable material, and a small amount of phenol red. The pH is brought to about 7.6

TABLE 1

AMINO ACID MIXTURE		SALT MIXTURE A	
	mgm.		*mgm.*
Glycine	5.0	NaCl	50.0
dl-Valine	10.0	K_2HPO_4	1.8
dl-Phenylalanine	1.0	$CaCl_2$	0.11
dl-Methionine	2.0	$MgSO_4 \cdot 7H_2O$	0.11
l-Glutamic acid HCl	50.0	$FeCl_3 \cdot 6H_2O$	0.035
l-Cystine	1.0		
l-Tryptophane	1.0		
l-Histidine HCl	1.0		

with sodium hydroxide. The volumes are then adjusted to 10 cc. per tube and the medium sterilized in the autoclave at 10 pounds pressure for ten minutes. Inoculation is made from a twenty-four hour broth culture of the organism and the tubes are incubated in a slanted position at 35° for about sixty hours. Determination of the total nitrogen of the bacterial growth is then carried out by the method earlier described (Mueller, 1935b).

EFFECT OF LIEBIG'S EXTRACT

If, to a control solution of amino acids, ethyl alcohol and salts, a solution of Liebig's extract of meat be added in varying proportions the effect on the growth of this strain of diphtheria bacillus is illustrated in figure 1.

However, as a starting point for the isolation of substances of an unknown nature, Liebig's extract offers certain quite evident drawbacks, notably the fact that the method of its preparation is not under immediate control and is, to a certain extent at least, not generally known. The heating to which the material is subjected during evaporation, quite irrespective of the nature of the substance extracted and the manner of extraction, may well lead to the production of a considerable number of by-products in addition to the numerous normal components of a tissue extract. It therefore seemed wiser to cast about for some other source of

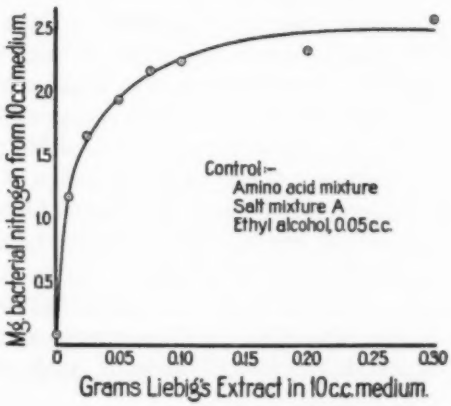

FIG. 1

material equally effective, free from the drawbacks pointed out above and if possible available in considerable quantities at moderate expense. We have considered, and in a superficial way tested the possibility of using plant extractives and although such extracts are efficacious we have discarded this source, for the present at least, as involving still other difficulties.

EFFECT OF FRESH TISSUE EXTRACTS

Animal tissue, of whatever source, appears to supply an active extract. An unexpected difficulty however, was met with, in that the use of considerable quantities of freshly prepared hot-water extract of muscle and other tissue caused an acid reaction

to develop in the cultures which stopped the growth at an early stage through direct inhibition by the pH.

It is probable, but by no means definitely proved, that this effect is due to glucose or other fermentable carbohydrate in the infusion. Thus, it has occasionally been possible to destroy the carbohydrate by fermentation with yeast, thereby avoiding the formation of acid, with consequent heavy growth. Such a procedure naturally introduces the possibility that the yeast itself furnishes an essential element, and is therefor not suitable for the purposes of our work. It was also possible in some experiments, so to alter the infusion by autoclaving with NaOH, which should destroy glucose, that heavy growth resulted. However, controls in which glucose was again added to such treated infusions and in a quantity comparable to that which must have been present in the original infusion have also occasionally permitted heavy growth. We are, therefore, not in a position to state definitely that this peculiarity is due to the presence of glucose, nor to explain why it is not met with in the case of Liebig's extract.

In general, extracts of various animal tissues behave in much the same way as does beef-heart infusion. For example, a boiled and filtered extract of beef liver showed marked growth-stimulating properties in small quantities, but in larger amounts produced an acid reaction which checked the growth, again probably due to the presence of glucose.

LIVER EXTRACT

Liver infusion offers an unusually desirable starting point for an investigation of this type, since it is now being actively studied by a number of workers in connection with its curative effect in pernicious anaemia. We were fortunate in enlisting the interest and assistance of Dr. Yellapragada Subbarow of the Department of Biochemistry at Harvard Medical School in our problem. He was able to supply us with various fractions of a liver extract which had been prepared originally according to his specifications following the method of Cohn, McMeekin and Minot (1933), and could presumably be closely duplicated. We found most of his fractions active in varying degree and were able to obtain

from him one fraction prepared from a ton of beef liver, of no use for his purposes, showing a quite satisfactory degree of stimulation of growth of the diphtheria bacillus. The work to be described below has been carried out with this fraction.

The method of preparation of this fraction as described to us by Dr. Subbarow is roughly as follows: Fresh liver is minced, extracted with water at 70°C. and filtered; the filtrate is concentrated in vacuum to a syrup and is precipitated by the addition of alcohol up to a concentration of 65 per cent and is again filtered. To the 65 per cent alcoholic filtrate more absolute alcohol is added

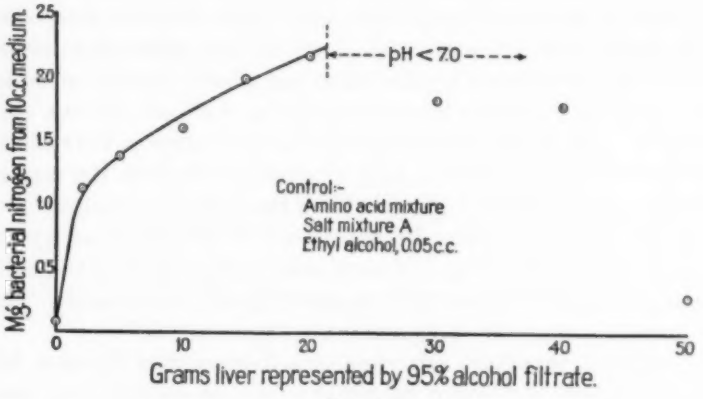

FIG. 2

to a concentration of 95 per cent and the precipitate is again removed. The 95 per cent alcohol filtrate, which has been concentrated in vacuum to a syrup is the material supplied us by Dr. Subbarow. The substances contained in this fraction are almost without effect in pernicious anaemia but show considerable growth stimulation for our strain of diphtheria bacillus. By no means all of the essential material occurring in liver is present in this fraction, but there is a fairly high concentration and it represents a by-product source which is a distinct advantage from the standpoint of expense; and at the same time it is prepared in a manner easily duplicated. In addition, a considerable degree of purification may be assumed to have taken place. Figure 2

shows the effect of the addition of this concentrate to the control solution.

It will be seen that even here one is faced with the problem of removing the acid-forming substances almost as a preliminary step in the investigation. Fortunately this proved to be rather easily accomplished.

The methods which appear to be producing the most promising results at present in the isolation of active substances from such mixtures depend upon adsorption and subsequent elution. Both charcoal or fuller's earth are available as adsorbents and suitable methods of elution for each have now been pretty well worked out. Moreover, Koser and Saunders (1935) have recently shown that adsorption of meat extract by charcoal with subsequent elution by alcohol or acetone yielded solutions which strongly stimulate the growth of certain bacteria including *Corynebacterium diphtheriae*. The writer (Mueller, 1922) in 1921 showed that adsorption of beef-heart infusion with charcoal resulted in the removal of factors necessary for the growth of the hemolytic streptococcus and the pneumococcus. Freedman and Funk subsequently confirmed this observation, and were able to elute most of the active material from the charcoal by means of glacial acetic acid. They believed the material to be similar to "vitamine B."

We have, therefore, adsorbed this liver-extract fraction with norit charcoal and eluted the latter with acid alcohol, very much according to a method outlined to us by Dr. Subbarow and almost identical with that used by Kinnersley, O'Brien, Peters and Reader (1933) in their work on vitamins B_1 and B_4.

Six hundred and fifty cubic centimeters of the syrupy concentrate of the 95 per cent alcohol filtrate of the liver extract, supplied us by Dr. Subbarow, and equivalent to approximately 100 kgm. of beef liver, were diluted to 5,000 cc. and precipitated by the addition of 200 cc. of 25 per cent neutral lead acetate. The precipitate was discarded, the lead removed with H_2S and the latter distilled out *in vacuo*. The filtrate was adsorbed by stirring at room temperature for twenty minutes with 200 gm. norit charcoal which had not been previously treated with acid to remove inorganic material. The charcoal was filtered and washed

with cold water; the filtrate was again adsorbed in the same way with a similar amount of norit. Both adsorptions were carried out without adjusting the pH of the solution which was, of course, fairly acid (4.5–5.0) as a result of the lead acetate precipitation. The filtrate from the second adsorption was concentrated *in vacuo* until crystallization commenced.

The combined washed charcoals were eluted three times as follows: First with 4 liters of 50 per cent alcohol containing 40 cc. of concentrated hydrochloric acid by boiling with constant stirring for fifteen minutes on the steam bath. Second, with 4 liters of 50 per cent alcohol containing 12 cc. of concentrated HCl by ten minutes of boiling and stirring. The third elution was a repetition of the second. The three elutes were mixed after filtration,

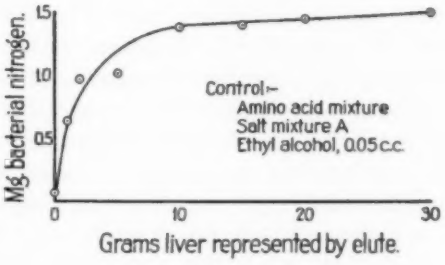

FIG. 3

and concentrated in vacuum to about 1,200 cc. Of this concentrate, 1 cc. is therefore equivalent to about 85 grams of liver.

Figure 3 demonstrates the effect of the addition of the adsorbed and eluted material to the control solution.

It is evident that one or more substances taking part in the growth-stimulating effect of the liver extract have been removed by charcoal and can again be obtained from it by the method of elution above described. The effect of this elute, however, is much less marked than the effect of the whole crude fraction of the liver extract. Tests carried out with the filtrate from the charcoal showed almost no growth-stimulating properties to be present in this material but, when it was mixed with the elute and again tested, a marked increase in growth over that produced

by the elute alone resulted, as shown in figure 3. However, the charcoal filtrate still contained the substances responsible for the development of an acid reaction and smooth curves could not be obtained. The actual figures for such experiments may therefore perhaps be omitted, but it was clear that there were at least two types of substance having an effect on the growth of our organism present in the liver-extract fraction. One class is readily adsorbed on charcoal and eluted from it by acid alcohol, the other class is not adsorbed by charcoal and is present in the filtrate.

It seemed desirable to investigate, first, the nature of the supplementing material present in the charcoal filtrate since it is clear that substances not readily adsorbed would be expected to withstand the usual chemical manipulations more readily than those which, being easily adsorbed, would probably suffer heavy losses on inert precipitates, etc.

The charcoal filtrate when concentrated in vacuum evidently contained considerable quantities of organic material and a large crop of crystals grossly resembling impure leucine separated. Since it is quite certain that various amino acids occur in tissue extracts, in small amounts at least, and since, because of the method of fractionation employed in the preparation of this material, they would almost surely be present in this fraction, it was assumed, to begin with, that there was still perhaps a deficiency of one or more amino acids from the control solution. This was a possibility which had been kept in mind in our earlier work on the amino acid requirements, since there was every reason to believe that Liebig's extract contained certain, at any rate, of these compounds. A total acid hydrolysate of casein prepared in the ordinary way was therefore substituted for the charcoal filtrate and it was found that quite as heavy growth resulted as in the case of the liver-extract fraction. For the sake of brevity this protocol, as well as some others which served only to point the direction, may be omitted.

The attempt was made in the usual manner to determine into which main group of the amino acids the new essential factor fell and results were obtained which were not clear cut, but which, after a time, suggested that we were not dealing with an amino

acid but rather with some quite different type of material, possibly only an inorganic salt.

Throughout the work, a mixture of inorganic salts containing the usual elements and groups had been supplied (salt mixture A). The proportions, however, were perhaps carelessly fixed upon, for it was felt that as long as tissue extractives in crude form were being supplied, the inorganic elements would, to a certain extent, be sufficiently well taken care of. Now, however, upon increasing tenfold the previously used concentrations of KCl, $CaCl_2$, and $MgSO_4$, growth with the usual amino acid control and the charcoal elute was quite satisfactorily heavy.

The optimal concentrations of the various inorganic constituents in the presence of the liver elute and the amino acid control have been determined as accurately as possible by means of curves which, for the sake of brevity, may be omitted. Should it become possible to substitute pure compounds for the liver elute which almost surely contains some inorganic material, it may be worth while to reinvestigate the salt requirements which could then be done with some accuracy. At present, as a result of a considerable number of experiments, a new salt mixture has been determined upon as giving excellent results. Probably the SO_4 and Fe may be omitted, but their presence makes little or no difference. There is some indication that the Fe is even slightly inhibitory, but the effect is so small as to be in doubt with the present set up. The effect of the sodium ion is also in doubt, its omission necessitating the use of KOH to adjust the reaction of the medium in rather considerable amounts because of the HCl present in the liver elute and in the hydrochlorides of glutamic acid and histidine. Growth in such media is poor, but whether on account of the lack of Na or the great excess of K cannot now be stated.

The presence of the Ca ion is apparently unnecessary, at any rate in excess of that which may be present in the liver elute. Since the charcoal was not acid-washed it is altogether probable that some calcium phosphate is derived from that source and would be present in the medium. Additional amounts of $CaCl_2$ however, lead to the formation of precipitates which apparently contain phosphates of both Ca and Mg and the growth is less

good than when Ca is entirely omitted. The precipitate forms, usually, during the autoclaving of the medium and may be for the most part avoided by the addition of sterile $CaCl_2$ by means of a sterile pipette after the medium has been autoclaved. Even with this technique, however, the growth is not so good as with the omission of the element.

TABLE 2

SALT MIXTURE B	
	mgm.
$NaCl$	50.0
KH_2PO_4	3.5
$MgCl_2 \cdot 6H_2O$	3.0
H_2SO_4	1.0
$FeCl_3 \cdot 6H_2O$	0.1

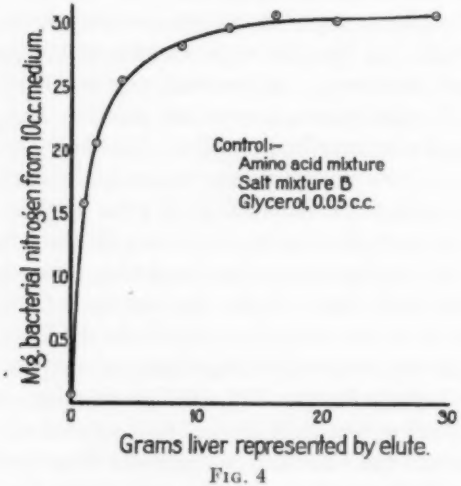

FIG. 4

The composition of the salt mixture as employed at present is as shown in table 2.

Figure 4 shows the effect of the addition of the liver elute to a control solution containing salt mixture B in place of salt mixture A and may therefore be compared directly with figure 3 as

illustrating the difference in effect produced by the increased concentration of the inorganic elements. It will be noticed that glycerol has been substituted for ethyl alcohol, which, however, should lead to no material difference in the growth. For various reasons, in the latter part of the work glycerol has been used routinely and its effect is apparently quite similar to that of the alcohol.

The striking effect of the liver elute produced by a relatively minute amount of material suggests, of course, something of the action of a vitamin-like substance. Certain components of the B Vitamin complex are almost surely present and obviously also, many other substances of still unknown composition are to be found in such a preparation. It is quite impossible at this time to state whether one substance or several will be found to be involved. It has been possible thus far to attempt to substitute crystalline vitamin B_1 obtained from Merck & Company, the *Staphylococcus* vitamin of Knight, a small specimen of which was kindly placed at our disposal by Dr. Knight, and a preparation of pantothenic acid, for which we are indebted to Dr. Roger J. Williams; none of these three substances by itself showed any appreciable growth-stimulating effect when substituted for the liver elute. This, of course, does not mean that one or all may not be involved along with additional compounds. Work on this question is now under way and it is hoped to be able to obtain more definite information to be reported later.

CONCLUSIONS

1. Animal tissue extracts, apparently of whatever source, contain substances essential to the growth of our strain of diphtheria bacillus.

2. There is also present in most freshly prepared tissue infusions a substance, perhaps glucose, which inhibits growth through the formation of acid.

3. An extract of beef liver has been found to be highly effective in stimulating growth of the organism and a considerable proportion of the effective material is present in a 95 per cent alcohol filtrate of an aqueous liver extract, which is available in con-

siderable quantities as a by-product in the preparation of the pernicious anaemia curative fraction.

4. One or more substances essential to growth may be removed from such a solution by means of adsorption on wood charcoal and may be recovered from the latter by elution with acid alcohol. The nature of the compounds here involved is still unknown but is being investigated.

5. The filtrate from such charcoal adsorptions also contains growth-stimulating materials which have been fairly well defined as inorganic in nature, probably for the most part K and Mg.

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STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

VII. AMINO ACID REQUIREMENTS FOR THE PARK-WILLIAMS NO. 8 STRAIN OF DIPHTHERIA

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It is a well-recognized fact that the nutritional requirements of an individual strain of any organism may be materially altered by prolonged cultivation on artificial media. Examples are so numerous and so well known that they need not be reviewed in detail. In many instances such changes are accompanied by alterations in the nature of the organism itself as in smooth to rough variation, while in other cases no such changes have been noted. The need of a particular organism for such an amino acid as tryptophane has been found to disappear after repeated subcultures on media with gradually diminishing concentrations of this amino acid (Fildes, Gladstone and Knight, 1933). The ability of organisms to grow on an exceedingly simple synthetic medium has been enhanced by the gradual substitution of such a solution for much more complex mixtures. Many other examples naturally suggest themselves.

The diphtheria group has been widely studied in connection with its growth requirements, perhaps more so than other varieties of bacteria because of the interest attached to the process of toxin formation. It is now well known that great differences

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exist between various strains of this organism. In the literature are to be found reports of strains which grow and even produce toxin on synthetic media of the type suggested by Braun (1928) or by Wadsworth and Wheeler (1934), of others which grow on the so called "semi-synthetic" media containing small amounts of peptones or tissue extractives, and of still others which do well only on the usual infusion peptone media. To what extent these variations were present originally in the particular strain of organism and to what extent they have been produced artificially by prolonged adaptation is a matter which remains still to be cleared up.

The writer has studied in some detail the requirements of a single strain of the diphtheria bacillus isolated in 1928 by Dr. Ho Yu (1930) and grown since that time on a peptone meat-infusion broth with short intervening periods on Loeffler's medium. This organism may conveniently be called the HY strain. The results of this study have been presented in earlier publications (Mueller, 1935). It seems now desirable to extend such studies to other strains of diphtheria bacilli and the most logical one to begin with would seem to be that which is used in most laboratories for the production of a toxin of high titer, namely the Park-Williams No. 8 strain. With the probability in mind that differences will be found to exist among cultures of this strain obtainable from various sources because of variations in conditions to which the organism has been subjected over a period of years, this study has been carried out with a single strain of the Park-Williams No. 8 obtained from the Massachusetts State Antitoxin Laboratory through the courtesy of Dr. E. S. A. Robinson early in 1934. It will naturally be extended to strains of the Park-Williams organism from other sources and if differences are found to exist they will be presented in due time. That an extension of the study will necessarily have to be made when further information is available concerning the nature of the substances involved in the tissue extractive fraction, goes without saying. However, since the amino acid requirements for the HY strain which has already been studied are complex, there

would appear to be sufficient basis at the present time for a comparison with other strains on the basis of these compounds.

METHODS EMPLOYED

The same procedure has been followed in studying this strain as that used heretofore and described in detail in earlier publications. The substances used have been dissolved in water, added in the required proportion to special centrifuge tubes, the pH adjusted to 7.4 to 7.6 and the volumes brought to exactly 10 cc. After sterilization for ten minutes at 10 pounds pressure, inoculation with a loopful of pellicle from a 24-hour broth culture, and incubation in a sloped position at 35°C. for 60 to 72 hours, the bacterial growth has been killed by heat, centrifuged and washed and the nitrogen determined by the Pregl micro-Kjeldahl method. Results are given in terms of such bacterial nitrogen and for purposes of orientation it may be stated that 0.8 to 1.0 mgm. nitrogen is produced under the same conditions by the same strain on 10 cc. of the usual unenriched meat infusion peptone broth, this representing satisfactory growth.

TISSUE EXTRACTIVE FRACTION

In place of a crude meat infusion or of Liebig's Meat Extract we have used in this study a preparation from beef liver which we shall call a "liver elute." The procedure followed in obtaining this material has already been described (Mueller, 1935). It consists briefly in precipitating a concentrated aqueous liver extract with alcohol up to 95 per cent, removing the alcohol from the filtrate, adsorbing with charcoal and eluting with acid alcohol. After concentration to remove alcohol, 1.0 cc. of this material is equivalent to 80 grams of liver, and contains 0.0475 gram solids dried at 110°. The solution itself will serve as a starting point for a further study of the substances involved. Undoubtedly many compounds are present in this fraction but it represents a considerable degree of purification over a crude infusion, by a method which should be susceptible to duplication.

AMINO ACID REQUIREMENTS

Preliminary experiments which need not be given in full indicated that our culture of the Park 8 strain grew badly on the same medium which permitted heavy growth of the HY strain of the diphtheria bacillus. Further experiments indicated that the fault lay, not with the tissue extractive fraction (liver elute) nor with the inorganic constituents, but with the amino acids. Certain of these substances required by the HY strain were evidently unessential, at any rate for first generation growth, and moreover it appeared that one or more amino acids were essential to the Park 8 strain for which the other strain had no requirements. By the usual process of separation of the amino acids into three groups by the Dakin butyl-alcohol extraction method, it was found that this new requirement was localized in the mono-amino monocarboxylic fraction. This fraction in turn, upon further examination, appeared to owe its additional activity entirely to its content of leucine, provided the amino acids used for the earlier strain were also present. It was now possible in a series of experiments to determine which of the other amino acids could be omitted without causing diminution in growth and the amino acids essential to heavy growth were in this way limited to valine, leucine, glutamic acid and cystine. The addition of methionine, while not essential for fairly heavy growth in the first generation, produced a marked increase in the quantity of nitrogen obtained, either with or without the concomitant presence of histidine. The latter substance had been found to play an apparently important rôle together with methionine in the study of the HY strain, but with this strain of the Park 8 it is evidently not essential.

The results may be most clearly expressed in a series of curves. For the first six of these the following composition was determined as giving satisfactorily heavy growth and controls were made in such a way that the indicated quantities of all but one substance were present in each tube. The material which was omitted was then added in increasing amounts with the idea of learning in this way the smallest amount necessary for maximal growth.

dl-Valine	10.0 mgm.
dl-Leucine	10.0 mgm.
d-Glutamic acid HCl	50.0 mgm.
l-Cystine	1.0 mgm.
Glycerol	0.05 cc.
Liver elute	0.25 cc.
Salt mixture*	

* The salt mixture had been determined upon in a preliminary series of experiments using both the Park 8 and the HY strain as test organisms. It is added in constant amounts, as indicated below, to each tube.

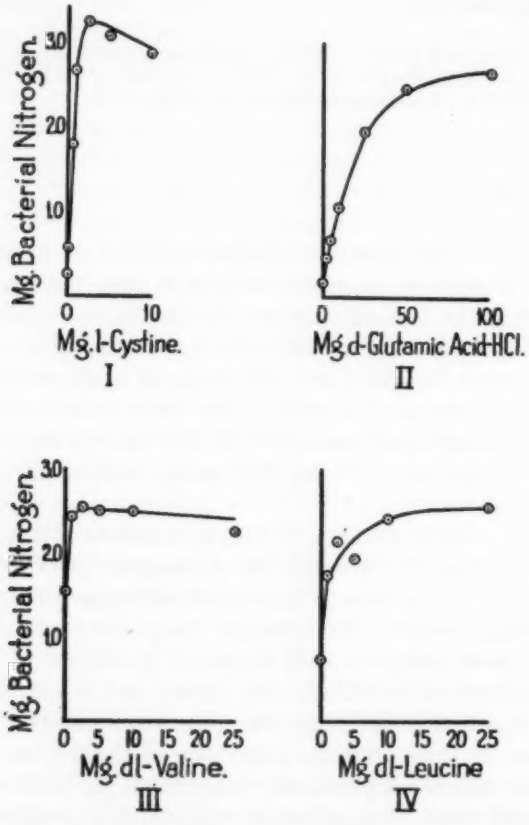
$NaCl$	50 mgm.
KH_2PO_4	3.5 mgm.
$MgCl_2 \cdot 6H_2O$	3 mgm.

In the seventh curve, increasing amounts of methionine are added to the above complete medium to show the further stimulating effect of this substance. In the eighth experiment the quantities of valine and cystine have been changed to 2.5 mgm. of each, since Charts I and III indicate such quantities to be optimal, 2.0 mgm. of dl-methionine have been added, and the effect of leucine again studied with this control medium, the results shown in curve IV for this amino acid being not entirely satisfactory.

With the exception of curve VII, in which duplicate tubes were not run, each point plotted is the average of two tubes.

The charts show clearly the growth-stimulative effect of each of the constituents of the medium except the inorganic portion. There is some evidence that excessive quantities of any of the materials become inhibitory, as, indeed one should expect, and that for optimal growth one must select suitable concentrations, which can be done from an inspection of the curves. The only substance calling for particular comment is glutamic acid. This amino acid must be supplied in considerably greater quantities than any of the others. It is unfortunately still impossible to determine whether its effect may not be due to an impurity rather than to glutamic acid itself. Neither sodium nor ammonium glutamate can replace it. Aspartic acid is without effect, and a specimen of dl-hydroxy glutamic acid supplied us through the

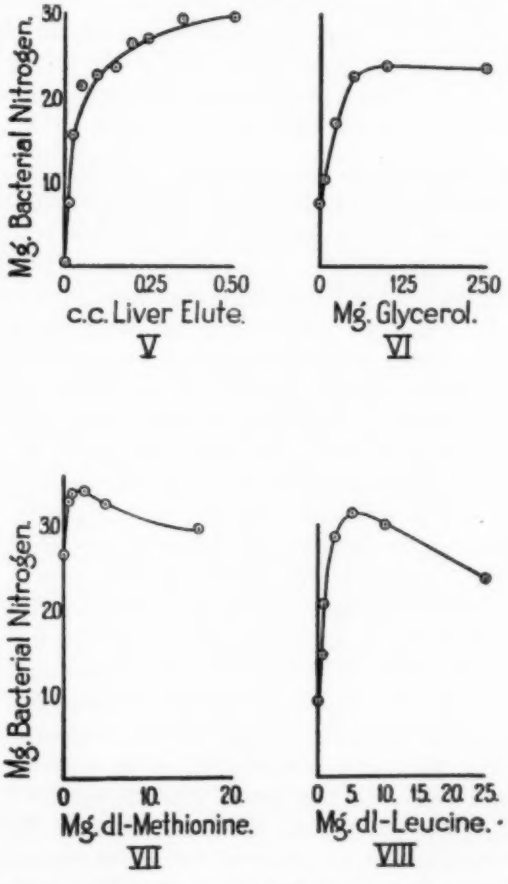
courtesy of Dr. Hans T. Clarke of the College of Physicians & Surgeons in New York was equally ineffective. Synthetic glutamic acid has not been available to us, and a definite answer to the problem will be possible only when this material can be



substituted for the natural product. We hope to be able to carry out such experiments in the near future. Indirect evidence that glutamic acid itself is involved is found in the experiments of Abt. (Abt, 1925). From a quantitative study of the ratio of CO_2 to NH_3 formed in growing cultures of the diphtheria bacillus,

this author concludes that glutamic acid is the amino acid metabolized in greatest quantity.

In all of the work described in this, as in our earlier reports dealing with the diphtheria bacillus, only first generation growth



resulting from a relatively heavy inoculum, has been dealt with. It seemed desirable to determine whether or not this organism could be passed through repeated subculture on such simplified media. A series of tubes were therefore prepared, each con-

taining 10 cc. of a medium of the following composition, the quantities being chosen from an examination of their several curves:

l-Cystine	2.5 mgm.
dl-Valine	2.5 mgm.
dl-Leucine	5.0 mgm.
d-Glutamic acid HCl	50.0 mgm.
dl-Methionine	2.0 mgm.
Liver elute	0.25 cc.
Glycerol	0.05 cc.
Salt mixture	

After sterilization in the autoclave, one tube was inoculated from the pellicle of a broth culture, incubated for 18 hours in a vertical position, and a loopful of the pellicle transferred to a second tube. The first tube was now slanted as usual in order to increase the surface, and incubation continued for a further period of 60 to 72 hours, at which time the bacterial nitrogen was determined. In this way, the organism was passed through seven consecutive daily transplantations on the above medium. The nitrogen in milligrams resulting in these tubes day by day was as follows: 1.93, 2.37, 2.68, 2.87, 2.93, 3.01 and 3.12.

It is clear that there is no tendency for the organism to diminish its growth on this medium. On the contrary, with adaptation, which takes place rapidly, the strain grows more and more luxuriantly. To explain the relatively poor growth in the first two generations as compared with the maxima of the curves and with the growth in the next few generations the following statement must be made: The preliminary studies and the curves were carried out with the strain of Park-Williams No. 8 obtained, as stated above, from the Massachusetts Antitoxin Laboratory more than a year ago, and carried since that time by daily transfer on a 2 per cent "proteose" peptone meat infusion broth. After completing the curves, which was done in Halifax during the summer, the work was interrupted, to be resumed in Boston. The Halifax strain was not brought back to Boston since the same original strain had been carried there on the same stock medium and under the same conditions. It appeared, however, that something had gone amiss with this strain, and it became neces-

sary to go back to the original Massachusetts Antitoxin Laboratory strain. The passage experiment was therefore carried out with this freshly acquired strain, which had been kept during the past fifteen or sixteen months by methods differing somewhat from those which we had used. Evidently the conditions which had been fixed as optimal for the strain as we carried it were not completely so for the strain when kept under different ones. That the changes involved were not deep-seated is indicated by the rapidity with which growth attained the same maximum indicated by the charts.

As a more exact explanation of what had occurred, it was found that increasing the quantity of dl-valine from 2.5 to 5 mgm. and adding 5 mgm. of glycine produced 3.30 mgm. bacterial nitrogen in the first generation. The slight change was evidently attributable to a variation in the ability of the organism to produce these two substances.

Comparison of the results obtained with the HY and the Park-Williams strains show distinct differences, as well as similarities. Fildes and Richardson (1935) have carried out similar studies with the *Clostridium sporogenes*, and find a somewhat similar list of amino acids, some of which they list as indispensable, others as necessary only for heavy growth. We have not attempted to separate the requirements in this way, but for the present group them all as necessary for heavy growth. The similarities and differences shown by the two strains of diphtheria and by the *Cl. sporogenes* may most easily be seen from the following tabulation:

Diphtheria (HY)	*Diphtheria (P-W. No. 8)*	*Cl. sporogenes*
Glycine	Valine	Leucine
Valine	Leucine	Tryptophane
Methionine	Methionine	Phenylalanine
Phenylalanine*	Cystine	Tyrosine
Tryptophane	Glutamic acid	Arginine
Cystine		Histidine
Histidine		Cystine
Glutamic acid		Methionine
		Valine

* Found in later work to have very little effect.

A general similarity is evident, but at the present stage of the study of bacterial cultural requirements one cannot predict how valuable a more complete knowledge of the facts will prove to be, either for purposes of classification and differentiation, or from the more general point of view of processes underlying the metabolism of amino acids. In the latter connection, such studies as those of Stickland (1934) on the oxidation-reduction mechanisms of amino acids in bacterial media are interesting and suggestive.

CONCLUSIONS

1. A strain of the Park 8 diphtheria bacillus was found to grow heavily on a medium containing only l-cystine, d-glutamic acid, dl-valine, dl-leucine, dl-methionine, glycerol, inorganic salts and "liver elute." The method of preparing the "liver elute" is described.

2. The medium is suitable for serial culture, the amount of growth increasing with adaptation.

3. Moderate variation in conditions under which the strain is routinely maintained may lead to differences in its amino acid requirements.

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A BIOLOGICAL CLASSIFICATION OF KLEBSIELLA OZENAE[1]

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The experience of most workers in clinical bacteriology has been that the organism isolated from certain conditions of the upper respiratory tract and commonly designated as "ozena bacillus" is so similar morphologically and culturally to comparatively numerous strains of Friedländer's bacillus that the two organisms are separable with difficulty, if at all. It is, therefore, the opinion of some that not only these organisms but even *Klebsiella rhinoscleromatis* should be regarded as identical. In connection with studies already published on the occurrence of different types of the Friedländer organisms (Julianelle, 1926) and on the immunological identity of the capsular antigens of all rhinoscleroma strains and Friedländer type C strains (Goslings, 1933; Morris and Julianelle, 1934), it was proposed to study the biological reactions of *Klebsiella ozenae* with the purpose of an attempted classification. This aim has been accomplished with a certain measure of success, and a report of the classification is made in the present report.

EXPERIMENTAL

Strains of bacteria studied

The bacteria studied in the present investigation included representative strains of types A, B, and C, Friedländer's bacillus, *Klebsiella rhinoscleromatis*, and 19 strains of *Klebsiella ozenae*. Of the latter strains, two were isolated in the otolaryngological

[1] Conducted under a grant from the Commonwealth Fund.

clinic of Washington University and the remainder were kindly sent by Dr. W. R. O. Goslings of Amsterdam, Netherlands, who

TABLE 1

Biochemical reactions of different strains of genus Klebsiella

ORGANISM	STRAIN	GLUCOSE	SUCROSE	MALTOSE	MANNITOL	LACTOSE	GELATIN	NITRATE REDUCTION	INDOL
Kleb. ozenae	04	AG	AG	AG	AG	AG	−	+	−
	05	AG	A	AG	AG	A	−	+	−
	06	AG	AG	AG	AG	AG	−	+	−
	011	AG	AG	AG	AG	AG	−	+	−
	013	AG	AG	AG	A	AG	−	+	−
	016	AG	AG	AG	AG	A	−	+	−
	022	AG	A	A	AG	A	−	+	−
	024	AG	AG	AG	AG	AG	−	+	−
	026	A	AG	AG	AG	AG	−	+	−
	027	AG	AG	AG	AG	AG	−	+	−
	028	A	AG	AG	AG	A	−	+	−
	029	AG	A±	AG	AG	AG	−	+	−
	030	AG	AG	AG	AG	AG	−	+	−
	01378	AG	AG	AG	AG	AG	−	+	−
	0M	A	AG	A	A	AG	−	+	−
Friedlander's bacillus	Type A-Sc	A	A	A	A	−	−	+	−
	Type A-F5	AG	AG	AG	AG	AG	−	+	−
	Type B-E	A	A	A	A	A	−	+	−
	Type B-F6	AG	AG	AG	AG	AG	−	+	−
	Type C-F10	AG	AG	AG	AG	AG	−	+	−
	Type C-M	AG	AG	AG	AG	AG	−	+	−
	Group X-F11	A	−	−	−	−	−	+	−
	Group X-F12	AG	−	AG	−	AG	−	+	−
Rhinoscleroma bacillus	R-8-3	A	−	A	A	−	−	+	−
	R-24-3	A	A delayed	A	A	−	−	+	−

A, indicates acid; G, gas; while + signifies a positive reaction; and −, no reaction.

isolated them from typical clinical cases chiefly in Sumatra, Dutch East Indies.

With these different strains, a study was made of biochemical reactions in different media, virulence, and immunological reac-

tions as determined by agglutination and agglutinin adsorption. The preparation of antisera and the technique of agglutination and agglutinin adsorption have been described in a previous communication (Julianelle, 1926).

BIOCHEMICAL REACTIONS

A number of differential media were inoculated in order to determine the biochemical activity of different strains of *K. ozenae*. For purposes of comparison, similar tests were made with representative cultures of *K. rhinoscleromatis* and with each type of Friedländer's bacillus. The media included the more commonly employed carbohydrates, gelatin, nitrate and peptone broth (for detection of indol formation). Cultures were incubated at 37°C. for one week when final observations were made. The reactions occurring in the different media have been summarized in table 1.

The fermentation reactions of the Friedländer and rhinoscleroma organisms have been commented on before (1922, 1930, 1934) and require no further discussion except to point out again the variability of the different strains of the former organism and the relative uniformity of the latter. In the case of *K. ozenae* the variability characteristic of Friedländer's bacillus is also apparent, demonstrating clearly that such a method of classification is unsatisfactory and uncertain. None of the strains liquefy gelatin or form indol, while reduction of nitrates to nitrites was detected in each instance. The data support observations already published which indicate that the biochemical reactions of the organisms of this genus do not serve for purposes of practical classification.

VIRULENCE

It has already been shown that types A and B of Friedländer's bacillus are virulent for both white mice and rabbits (1926 a, b). Type C strains and rhinoscleroma strains, on the other hand, (1934) are avirulent, even after repeated attempted passage through white mice. Eight of the ozena strains selected at random were tested for virulence in white mice by intraperitoneal

injection of 0.5 cc. of eight- to ten-hour broth cultures. Seven of these strains were not fatal in the quantity indicated even after several intraperitoneal passages, but the remaining culture (O_{28}) killed mice within twenty-four hours. With this strain, moreover, it was possible to increase the initial virulence so that 0.01 cc. of broth culture killed within twenty-four to forty-eight hours. It was not possible, however, to increase the fatal dose beyond this point by mouse passage, and it was found, on the contrary, that with subculture virulence rapidly decreased to its original titer. The indications are, therefore, that, like both type C (Friedländer) and rhinoscleroma strains ozena cultures are lacking in virulence. An occasional strain may be found capable of killing mice, but in relatively large amounts of culture (10^{-1} cc.) as compared with types A and B Friedländer's bacillus (10^{-6} and 10^{-7} cc.), so that for practical purposes virulence of the ozena organisms may be disregarded.

AGGLUTINATION REACTIONS

Agglutination tests were performed with all 19 strains of *K. ozenae* in rabbit antisera of 9 strains. The specific agglutinin titer of the antisera was not particularly high and varied from 1:80 to 1:320. All antisera, however, contained varying quantities of species-specific antibodies reaching to 1:5000 and more in some instances. Since this report, however, is concerned only with the capsular or type-specific reactions, the somatic or species-specific reactions will be reserved for discussion in a future publication. In order to facilitate analysis and comment, the agglutination reactions occurring in dilution of 1:10 of the sera are summarized in table 2. It will be observed that the majority of the strains (12) fall into one type. A second type comprises two strains, while the remaining strains (5) are heterogeneous and form a non-specific group. The type including the larger number of strains will be designated tentatively as type I, and the second as type II, while the heterogeneous strains may be designated as group III.

Immunological experiments in the past with organisms of this genus have revealed that certain strains (type B) of Friedländer's

bacillus cross react with type II Pneumococcus (Avery, Heidelberger and Goebel, 1925; Julianelle, 1926), with *Klebsiella genitalium* (Julianelle, 1926; Edwards, 1928), and *Bacterium aerogenes* (Edwards, 1929), and that type C strains cross react with *Klebsiella rhinoscleromatis* (1933). It was important, therefore, that the specificity of ozena strains should be determined by agglutination reactions with other cultures. These included the fixed types of Friedländer's bacillus, *Klebsiella rhinoscleromatis*, Pneumococcus

TABLE 2

Cross agglutination reactions with different strains of Klebsiella ozenae

STRAIN	ANTISERUM								
	1	1378	26	30	27	28	29	10-3	M
1	++++	++++	–	–	–	–	–	–	–
4	++++	++++	–	–	–	–	–	–	–
5	++++	++++	–	–	–	–	–	–	–
6	++++	++++	–	–	–	–	–	–	–
11	++++	++++	–	–	–	–	–	–	–
13	++++	++++	–	–	–	–	–	–	–
16	++++	++++	–	–	–	–	–	–	–
20	++++	++++	–	–	–	–	–	–	–
22	++++	++++	–	–	–	–	–	–	–
24	++++	++++	–	–	–	–	–	–	–
1378	++++	++++	–	–	–	–	–	–	–
W.U.	++++	++++	–	–	–	–	–	–	–
26	–	–	++++	++++	–	–	–	–	–
30	–	–	++++	++++	–	–	–	–	–
27	–	–	–	–	++++	–	–	–	–
28	–	–	–	–	–	++++	–	–	–
29	–	–	–	–	–	–	++++	–	–
10-3	–	–	–	–	–	–	–	++++	–
M	–	–	–	–	–	–	–	–	++++

The agglutinin titer of the different sera varied from 1:80 to 1:320. The reactions given are those observed in dilutions of 1:10.

type II, and a strain of *Klebsiella granulomatis* which was originally isolated from a patient with granuloma inguinale. While tests were done with all the ozena strains, it has been found simpler in arranging a protocol to record the results obtained with a few representative strains only, since the behavior of all the cultures was uniform. The data, as will be seen from table 3, indicate that ozena organisms do not cross react with antisera of the organism tested, and that antiozena sera do not agglutinate heterologous bacteria.

TABLE 3

Specificity of agglutination reactions of Klebsiella ozenae as demonstrated by cross reactions of different Klebsiella organisms

STRAINS	ANTISERA										
	Ozena					Friedländer			Rhinoscleroma strain 8-3	Pneumococcus Type II	Granuloma strain G2
	1	1378	26	28	M	Type A	Type B	Type C			
Ozena:											
1	++++	++++	−	−	−	−	−	−	−	−	−
1378	++++	++++	−	−	−	−	−	−	−	−	−
26	−	−	++++	−	−	−	−	−	−	−	−
28	−	−	−	++++	−	−	−	−	−	−	−
M	−	−	−	−	++++	−	−	−	−	−	−
Friedländer:											
A	−	−	−	−	−	++++	−	−	−	−	−
B	−	−	−	−	−	−	++++	−	−	++++	−
C	−	−	−	−	−	−	−	++++	++++	−	−
Rhinoscleroma 8-3	−	−	−	−	−	−	−	++++	++++	−	−
Pnc. II	−	−	−	−	−	−	++++	−	−	++++	−
Granuloma G2	−	−	−	−	−	−	−	−	−	−	++++

TABLE 4

Cross adsorption tests with different strains of Klebsiella ozenae

STRAIN OF OZENA	ANTI-OZENA SERUM											
	01378, type I							026, type II				
	Unadsorbed	Adsorbed with						Unadsorbed	Adsorbed with			
		04	016	026	027	R*	E*		04	016	026	027
04, type I	++++	−	−	++++	++++	++++	++++	−		Not done		
016, type I	++++	−	−	++++	++++	++++	++++	−		Not done		
026, type II	−			Not done				++++	++++	++++	−	++++
030, type II	−			Not done				++++	++++	++++	−	++++
027, group III	−			Not done				−		Not done		

* R is a strain of rhinoscleroma, and E of type B, Friedländer. Reactions are those in 1:5 dilution of antiserum.

AGGLUTININ ADSORPTION

The results of the agglutination reactions reported above indicate that cultures of *Klebsiella ozenae* are separable into two types and a heterogeneous group. Further evidence of the specificity of the types was sought for in agglutinin adsorption tests. The results of these tests are recorded in table 4. An examination of this table reveals that the agglutinins contained in antiozena sera are removed only by adsorption with strains of homologous types. Thus, agglutinins in antisera 01378 (type I) are adsorbed by strains O_4 and O_{16} (both type I) but not by O_{26} (type II) and O_{27} (group III), nor by strains of rhinoscleroma or Friedländer organisms (type B). Similarly, antisera O_{26} (type II) are deprived of agglutinins only when adsorbed with strains O_{26} (type II) and not with strains O_4, O_{16} (type I), or O_{27} (group III). The evidence, from agglutination, that *K. ozena* is divisible into two specific types and a group of heterogeneous strains is thus verified by adsorption tests.

DISCUSSION

An attempt to classify the organisms commonly referred to as ozena bacilli is reported in the present communication. A study of the more usual cultural reactions such as fermentation of sugars, formation of indol, reduction of nitrates, and gelatin liquefaction, reveals that a classification on this basis is unreliable and therefore useless. Similarly it was found that virulence does not serve for this purpose, since the strains tested are nonpathogenic for white mice.

By agglutination, however, it has been possible to define two specific types and a group of heterogeneous strains in a total of 19 cultures of *K. ozenae*. The type-specificity is sharply demarcated, so that no cross reactions occur in antisera of closely related organisms as Friedländer, rhinoscleroma, or granuloma bacilli, nor in antisera of type II Pneumococcus. By adsorption tests conducted with antisera of homologous and heterologous types the specificity of the different strains of *K. ozenae* is demonstrated conclusively.

SUMMARY AND CONCLUSIONS

1. A biological study has been made of 19 strains of *Klebsiella ozenae*.

2. All the strains were characterized by an inability to form indol or to liquefy gelatin, while all were able to reduce nitrates.

3. The reactions of the cultures in different sugar media disclose a variability in fermentation which precludes a possible classification on this basis.

4. The virulence of these organisms is of a low order or completely lacking.

5. The agglutination reaction detects two specific types among the 19 cultures of *Klebsiella ozenae*, one composed of 12 strains and the other of two strains, while the remaining 5 strains form a heterogeneous group.

6. Agglutinin adsorption tests confirm the results observed by agglutination.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW YORK CITY BRANCH

FIFTH MEETING, OCTOBER 8, 1935

THE MICROBIOLOGY OF THE MOUTH. *Henry A. Bartels*, Department of Oral Pathology, School of Dental and Oral Surgery, Columbia University, New York City.

The limiting of the bacterial population of the mouth by such forces as antibiosis, lysozyme, desquamation of the oral epithelium, mouth currents, and mechanical action of saliva and food bolus were discussed.

Local and systemic etiological hypotheses of dental caries were presented. Carbohydrate fermentation by *Lactobacillus acidophilus*, its presence and absence in carious and non-carious mouths was considered. The effects of deficient diets, size of corn and rice particles in adequate diets and endocrine dysfunction in dental caries production in animals and children were mentioned.

The rôle of fuso-spirochetal organisms in production of disease was discussed, emphasizing findings of Smith and Prake and Sayer that symbiotic relationship of fusiform bacillus, a vibrio, a spirochete and an anaerobic streptococcus was required to produce lesions. Effects of deficient diet or injection of scillaren B in animals with lesion production was mentioned. Illustrations of unusual morphological types of Vincent's organisms or closely allied fungi were shown.

Actinomycotic-like organisms, their significance in production of salivary stones and desquamative gingivitis and the relation of leptothrix to tartar formation was presented.

Occurrence of an ameba and a trichomonad in some pyorrhetic mouths and their possible rôle as scavengers was considered.

DISSIMILATION OF GLUCOSE BY HETEROFERMENTATIVE LACTIC ACID BACTERIA[1]

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It is convenient to divide the true lactic acid bacteria into two natural groups for study of their dissimilative properties. Orla-Jensen (1919) suggested that those bacteria which convert carbohydrates essentially into lactic acid be distinguished from those producing, in addition, substantial quantities of volatile acid and carbon dioxide. Kluyver and Donker (1924) proposed the terms homofermentative and heterofermentative respectively for the two groups.

Study of the intermediary dissimilation by the lactic acid bacteria has been confined for the most part to the homofermentative group and little attention has been given to the heterolactic forms.

In the present paper a scheme for the dissimilation of glucose by the heterofermentative bacteria is proposed based on the results of quantitative investigations carried out with several representative species.

Gayon and Dubourg (1901) have studied the dissimilation of glucose by the heterofermentative lactic acid bacteria. Although descriptions of the organisms were given, no attempt was made to classify or name them. Acetic, lactic, succinic and carbonic acids, ethyl alcohol and glycerol were identified as final products of fermentation. Equations were presented for the formation of the products from glucose. A secondary breakdown of lactic to acetic acid was also suggested. Kayser (1904), Laborde (1904), and Smit (1913) identified the same products found by Gayon

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and Dubourg in the fermentation of glucose by members of the heterofermentative group. Fred, Peterson and Davenport (1920) and Peterson and Fred (1920) determined products of the dissimilation of glucose by *Lactobacillus pentoaceticus*. They confirmed the reports of a secondary breakdown of intermediately-formed lactic acid but did not report the presence of succinic acid or glycerol. Pederson (1929a) quantitatively studied the products formed by several species of *Lactobacillus* and *Leuconostoc*. Interpretation of his results is made difficult by the failure to determine carbon dioxide and the other products on the same fermentation. Charleton, Nelson and Werkman (1934) presented a carbon balance of the dissimilation of glucose and of levulose by *Lactobacillus gracilis* and a new species which they named *Lactobacillus fructivorans*.

METHODS

Lactobacillus lycopersici, *Lactobacillus mannitopoeus* and *Lactobacillus acidophil-aerogenes* used in this experiment, were kindly furnished by Dr. C. S. Pederson of the New York Agricultural Experiment Station, Geneva, New York and were chosen as typical of the heterofermentative lactic acid bacteria. Detailed descriptions of these strains of *L. lycopersici* and *L. mannitopoeus* are given by Pederson (1929). The culture of *Lactobacillus acidophil-aerogenes* is a transplant of the original strain isolated from feces and described by Torrey and Rahe (1915). At the time of isolation the organism was described as producing hydrogen. In the present work no free hydrogen was detected.

The medium (table 1) consisted of 2 per cent glucose, 0.3 per cent yeast extract (Difco), 1.0 per cent peptone and 0.2 per cent di-basic potassium phosphate. The glucose was sterilized separately for twenty minutes at 20 pounds and added to the basal medium at the time of inoculation. One liter of medium was placed in each 2-liter flask and inoculated with 25 cc. of a three-day culture grown in medium of the same composition.

In later experiments (table 2) the phosphate content was changed to 0.6 per cent K_2HPO_4 and 0.6 per cent KH_2PO_4 and the pH was adjusted to 6.2. More rapid fermentation occurred

in this medium. The solutions of (*a*) glucose, (*b*) yeast extract and peptone, and (*c*) phosphates were sterilized separately for twenty minutes at 20 pounds pressure and combined just prior to inoculation.

Strict anaerobiosis was maintained by continuously bubbling oxygen-free nitrogen through the medium.

Methods of analysis

The fermented media were analyzed after incubation for three weeks at 30°C.

The carbon dioxide evolved by the fermentation was forced through Bowen potash bulbs by a stream of oxygen-free nitrogen and was determined gravimetrically. The residual carbon dioxide was determined by acidifying an aliquot part of the medium and heating under a reflux condenser with a stream of carbon-dioxide-free air passing through the liquid to carry the carbon dioxide into Bowen potash bulbs for gravimetric analysis.

The volatile acids were determined by steam-distilling two liters of distillate from 400 cc. of the fermented medium. The acids present in the distillate were determined by the partition method of Osburn, Wood and Werkman (1933). The non-volatile acids were determined on a 100 cc. aliquot part of the fermented medium. The acids were extracted with ethyl ether and the lactic acid determined by the Friedemann, Cotonio and Shaffer (1927) method. The succinic acid was determined on an aliquot part of the extract by preparation of the silver salts.

The ethyl alcohol was determined on 300 cc. of the medium by the method of Stahly, Osburn and Werkman (1934).

The sugar determinations were made on the medium at the time of inoculation and at the conclusion of the fermentation by the method of Munsen and Walker (1906) after deproteinating with basic lead acetate.

Accurate determination of glycerol is difficult because of the necessity of separating it from interfering substances. An aliquot part of the medium was evaporated to 15 cc. and enough NaOH added to insure alkaline extraction. This solution was taken up in sodium sulfate. A three-day extraction with ethyl

ether was necessary to obtain quantitative separation of glycerol from the medium. The ether extract was made up to a volume of 100 cc and glycerol determined by Wagenaar's (1911) method. Rather than allow the alkaline copper sulfate solution to stand several hours, as described in the original method, the precipitate was centrifuged and the analysis made on the supernatant liquid.

Purity of the cultures was insured by microscopic and cultural examination at the time of inoculation and just before analysis.

EXPERIMENTAL

In tables 1 and 2 may be found seven typical experiments showing the quantitative dissimilation of glucose by *L. mannitopoeus*, *L. lycopersici* and *L. acidophil-aerogenes*. Reliability of the experimental data is satisfactory in view of the difficulty encountered in making quantitative determinations of the end products. The general agreement between the carbon balances and the oxidation-reduction ratios is satisfactory. The data, therefore, should lend themselves to the formulation of a scheme for the dissimilation of glucose by typical lactic acid bacteria of the hetero-type.

In table 1, glycerol was not experimentally determined, and the results showed a low carbon recovery and an excess of oxidized products. From these data it was evident that some reduced product had not been determined. The work of Gayon and Dubourg (1901) and of Smit (1913) suggested that this reduced compound was glycerol. It has been found (table 2) under our conditions that the quantity of glycerol formed is equivalent to twice the acetic acid. If glycerol is calculated on this basis for the experiment shown in table 1, the carbon balances and oxidation-reduction ratios show excellent agreement. In a similar manner, if glycerol is calculated in the experiments of Peterson and Fred (1920) a more satisfactory agreement of their carbon and oxidation-reduction balances is obtained. Similarly calculation of glycerol from the data of Charleton, Nelson and Werkman (1934) improves the carbon and oxidation-reduction balances.

The small quantity of succinic acid present (table 2) suggests its origin from protein. *L. lycopersici* (table 2) shows a carbon

TABLE 1

Anaerobic dissimilation of glucose by L. mannitopoeus and L. lycopersici

PRODUCT	L. MANNITOPOEUS		L. LYCOPERSICI	
	mM *per liter*	mM *carbon*	mM *per liter*	mM *carbon*
Glucose fermented	64.4	386.4	51.0	306.0
Ethyl alcohol	42.8	85.6	30.25	60.5
Acetic acid	13.8	27.6	9.3	18.6
Carbon dioxide	58.0	58.0	38.0	38.0
Lactic acid	54.2	162.6	48.05	144.15
Glycerol*	27.6	82.8	18.6	55.8
Total		416.6		316.8
Percentage of carbon		107.4		103.5
$\frac{\text{Oxidation}}{\text{Reduction}}$ ratio		1.02		0.974

* Calculated.

TABLE 2

Anaerobic dissimilation of glucose by L. acidophilaerogenes and L. lycopersici

PRODUCTS	D. ACIDO-PHILAERO-GENES	L. LYCOPERSICI			
		I	II	III	IV
	mM *per liter*	mM *per liter*	mM *per liter*	mM *per liter*	mM *per liter*
Glucose fermented	88.9	80.0	92.1	102.2	112.2
Ethyl alcohol	39.5	63.0	69.6	74.1	78.9
Acetic acid	10.2	12.1	16.5	15.5	19.5
Carbon dioxide	43.9	64.3	80.0	81.0	95.1
Lactic acid	108.2	65.2	67.6	83.1	89.7
Glycerol	21.7	25.0	36.2	32.6	43.5
Succinic acid	0.8	3.8			
Percentage of carbon recovered:					
With succinic acid	100.2	104.2			
Without succinc acid	100.0	101.0	101.9	99.0	103.0
$\frac{\text{Oxidation}}{\text{Reduction}}$ ratio	.873	.851	.912	.884	.943

balance of 104 per cent when succinic acid is included and of 101 per cent when excluded. *L. acidophil-aerogenes* shows the carbon

recovered inclusive of succinic acid to be 100.2 per cent and, excluding succinic acid, to be almost exactly 100 per cent.

Both experiments show a small deficiency of oxidized products, although the error is within the experimental limits of the analysis of such a mixture.

In view of the lack of evidence to support any scheme of initial breakdown of glucose by the heterofermentative lactic acid bacteria, the scheme presented here will deal only with the final reactions of the dissimilation.

Peterson and Fred (1920a) have presented evidence for the formation of acetaldehyde as an intermediate compound in the dissimilation of glucose by *L. pentoaceticus*. The authors have been unable to secure acetaldehyde by fixation methods in sufficient quantity to characterize by its derivatives. It is not improbable that acetaldehyde formed is so readily utilized that the fixatives are unable to act or the organisms may be able to utilize the acetaldehyde in the presence of fixatives.

It is difficult to account for the formation of ethyl alcohol, assuming a splitting of the glucose into two 3-carbon compounds, and not postulate acetaldehyde as an intermediate precursor. The ethyl alcohol might arise: (*a*) from the acetaldehyde acting solely as a hydrogen acceptor, (*b*) from a Cannizzaro reaction between two molecules of acetaldehyde, forming both ethyl alcohol and acetic acid, or (*c*) from a mixed Cannizzaro reaction between acetaldehyde and some other aldehyde. In view of the absence of an oxidation product typical of a Cannizzaro reaction and equivalent in quantity to ethyl alcohol, it is highly probable that the latter is formed by direct reduction of acetaldehyde.

Peterson and Fred (1920) have shown that a sufficient quantity of acetic acid is formed by a slow secondary breakdown of lactic acid to account for that which occurs in the normal fermentation of glucose. It has been found in this laboratory that pyruvic acid is formed as an intermediate product in the dissimilation of lactic acid. Nelson and Werkman (1935) have further shown that pyruvic acid is fermented anaerobically by *L. lycopersici* with the production of acetic acid, carbon dioxide and lactic acid in equimolar quantities. One molecule of pyruvic acid is broken down to

acetic acid, carbon dioxide and active hydrogen while a second is reduced to lactic acid. These facts indicate that the acetic acid found in the breakdown of glucose probably arises from a secondary fermentation of lactic through pyruvic to acetic acid, carbon dioxide and active hydrogen. The evidence is that hydrogen acceptors which are not present when pyruvic acid alone is fermented are formed from glucose and reduced by the active hydrogen.

Peterson and Fred (1920) have shown the dissimilation of lactic to acetic and carbonic acids by *L. pentoaceticus*, but have shown no reduction product to account for the activated hydrogen in the reaction. Davis (1933) and Bertho and Glück (1932) have demonstrated the formation of hydrogen peroxide, equivalent to the oxygen used, by members of the homofermentative group. Hunt (1933) has shown by respiration experiments that a quantity of oxygen equivalent to the carbon dioxide produced was consumed by *L. pentoaceticus* in a fermentation using lactic acid as a substrate. In flask experiments he found little difference in the quantity of acetic acid formed under aerobic and anaerobic conditions. Hunt also did not account for the hydrogen formed in the reaction. It is clear that oxygen may act as an acceptor with the formation of hydrogen peroxide. Since the organisms are catalase-negative the H_2O_2 accumulates and may be determined quantitatively. Anaerobically there is some hydrogen acceptor other than oxygen functioning. Kluyver (1933) has suggested that the glycerol formed may account for the hydrogen liberated in the dissimilation of lactic acid. This view is supported by the data in Table 2. The quantity of glycerol produced is equivalent to the hydrogen that would be formed if acetic acid arose solely from the dissimilation of lactic acid. It appears that under the conditions of the experiment glyceric aldehyde or a closely related compound accepts the hydrogen formed by the secondary fermentation of the lactic acid with the formation of glycerol.

Peterson and Fred (1920) suggested equation 1 to account for the dissimilation of glucose by *L. pentoaceticus*. The deficiency in lactic acid obtained in their data was explained on the basis of

(1) $C_6H_{12}O_6 \longrightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2$

a secondary decomposition of lactic acid to acetic acid and carbon dioxide. While the values obtained by them support this view, the reaction cannot be considered general for the heterofermentative lactic acid bacteria—not even for *L. pentoaceticus* as will be apparent when the data of Pederson (1929a) are calculated on the basis of millimols of the various products.

Evidence indicates that a complex and unstable equilibrium exists among the products in a fermentation system, which may be shifted in one direction or another by a change in such conditions as composition of the medium, pH, or degree of aerobiosis or anaerobiosis. This shifting in the quantitative relationships of the products would not be accommodated by equation 1 since it requires the formation of lactic acid, ethyl alcohol, and carbon dioxide in equimolar concentrations. It is necessary to use a scheme that will account for the differences in the results as found by different investigators.

A tentative scheme for the dissimilation of glucose by the heterofermentative lactic acid bacteria is presented in figure 1. This scheme is flexible and will serve to explain the data presented in contributions on the dissimilation of glucose by the heterofermentative lactic acid bacteria. It is not complete in as much as the initial phases of phosphorylation are far from clear, and there is no convincing evidence as to the identity of the intermediary compounds. It is tempting to suggest the importance of phosphoglyceric acid; however, the compound has not been isolated and identified in these fermentations.

The values presented in the scheme are taken from data in table 2.

The scheme suggests two sources of carbon dioxide in the dissimilation of glucose: 1. A quantity of carbon dioxide equivalent to the ethyl alcohol is formed by the splitting of the hypothetical 3-carbon compound into acetaldehyde, carbon dioxide, and active hydrogen. 2. Carbon dioxide equivalent to acetic acid is formed from the secondary fermentation of lactic acid. The total quantity of carbon dioxide produced should, therefore, be equal to the sum of the mM of ethyl alcohol and acetic acid. The data

for *L. acidophil-aerogenes* in table 2 show 43.9 mM of carbon dioxide found as compared with the calculated value of 49.7 mM (sum of the mM of acetic acid and ethyl alcohol). The difference is only 5.8 mM. In adapting these values to the scheme, the number of mM of acetic acid determined (10.2) has

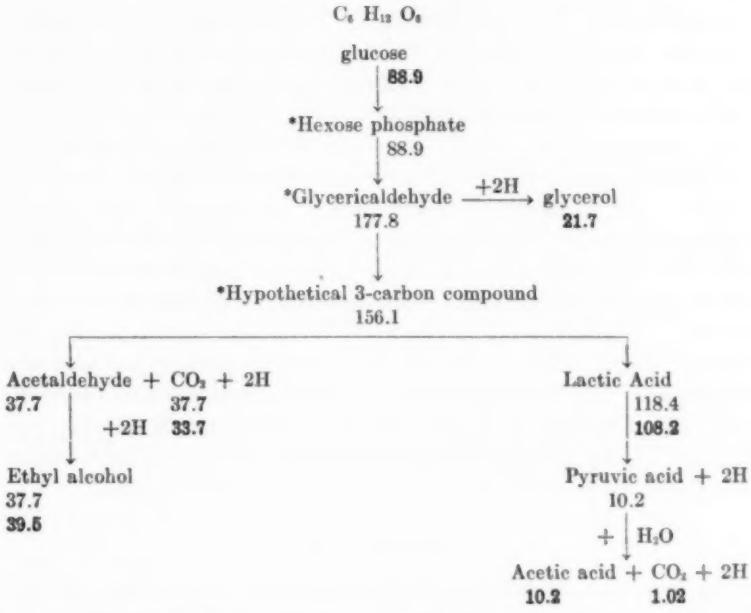

FIG. 1. SCHEME FOR THE DISSIMILATION OF GLUCOSE BY THE HETEROFERMENTATIVE LACTIC ACID BACTERIA

Figures in bold face represent mM found; other data are calculated.
* Has not been identified in heterofermentative lactic acid dissimilation.

been assigned to the carbon dioxide formed from the dissimilation of the lactic acid, since carbon dioxide and acetic acid are formed in equimolar quantities. The 5.8 mM difference between carbon dioxide determined and calculated is assigned to the carbon dioxide arising from the breakdown of the hypothetical 3-carbon compound.

It is possible to calculate the amount of glucose used from the products determined. The value thus obtained is 89.7 mM; the amount actually fermented was 88.9 mM.

By applying the experimental data to the scheme, the oxidation-reduction relationships may be more clearly pointed out. Active hydrogen may be formed from three sources: (*a*) the breakdown of the hypothetical 3-carbon compound to acetaldehyde, carbon dioxide and hydrogen, (*b*) the oxidation of lactic to pyruvic acid, and (*c*) the dissimilation of pyruvic acid to acetic acid, carbon dioxide and hydrogen. On the other hand there are only two points at which reduction takes place; the reduction of glyceric aldehyde to glycerol and of acetaldehyde to ethyl alcohol. In analyzing the data it will be seen that 20.4 mM of hydrogen were formed by the dissimilation of lactic acid and 39.5 mM from the breakdown of the hypothetical 3-carbon compound, totalling 59.9 mM of active hydrogen formed during the fermentation. It will readily be seen that 39.5 mM are used in the formation of ethyl alcohol, leaving 20.9 mM to reduce the glyceric aldehyde to (21.7 mM) glycerol. This analysis supports the view that the quantity of glycerol formed during the anaerobic fermentation of glucose by the heterfermentative lactic acid bacteria is equivalent to twice the quantity of acetic acid formed.

CONCLUSIONS

Carbon and oxidation-reduction balances for the fermentation of glucose by three species, representative of the heterofermentative lactic acid bacteria, are presented. Lactic, acetic and carbonic acids, ethyl alcohol and glycerol were found as end products.

The millimols of carbon dioxide produced were equivalent to the sum of the acetic acid and ethyl alcohol.

The glycerol produced was equivalent to twice the millimols of acetic acid.

A scheme is suggested for the dissimilation of glucose by the heterofermentative lactic acid bacteria.

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ANAEROBIC BACTERIA CAPABLE OF FERMENTING SULFITE WASTE LIQUOR

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Sulfite waste liquor (s.w.l.) results from the manufacture of cellulose pulp by the acid-sulfite process. It contains about 11 per cent of material, in solution, which consists of soluble lignin-sulfonic salts of calcium (60 to 70 per cent), hexose sugars (12 to 20 per cent), pentosans and pentoses (4 to 5 per cent), their various oxidation products, acetic and formic acids (0.5 to 1.0 per cent), and traces of other organic substances. When discharged into small natural waterways it frequently causes serious trouble, due primarily to its high biochemical oxygen demand and to the carbon dioxide evolved in fermentation of its sugars, which stimulates a prolific growth of algae.

The anaerobic fermentation of s.w.l. by bacteria of the sea-bottom mud (mixed cultures) studied by Benson and Partansky (1934), and by bacteria of river muds, studied by Partansky (unpublished data), shows that in the course of two to three months at 36°C. practically all organic matter of s.w.l. except lignin is converted into gases, and that the residue is a biochemically stable liquid. If the fermentation were to proceed throughout at the maximum rates attained in these mixed cultures, it would be complete in three to four weeks. It was concluded, therefore, that by using selected cultures and maintaining optimum conditions it should be possible to reduce the fermentation time to a matter of days, in which case it would be commercially possible to stabilize the s.w.l. before its discharge into the waterways, thus avoiding the pollution. It has been shown by us (1935) that various species of bacteria attack s.w.l. to only a

limited extent, and that bacteria fermenting s.w.l. can be isolated from sea-bottom and river-bottom muds. The search for organisms, actively fermenting s.w.l., the optimum conditions for the fermentation, and the study of the products of fermentation, was the object of this work. The present paper deals only with a description of the organisms isolated; the other phases will be the subject of a later communication.

ISOLATION OF THE ORGANISMS

Muds from several streams, lakes and other waterways, and a sample of garden soil (twelve different sources in all) were collected and placed in gallon bottles. The bottles were filled one-third full of mud and 850 cc. of neutralized s.w.l. was added. The container was then filled to the top with tap water and incubated at 36°C. When fermentation, as indicated by gas evolution, reached a maximum rate (about 500 to 600 cc. per day per gallon bottle) bacteria were isolated by dilution plating.

Four isolations were made from the three bottles in which the most rapid fermentation occurred.

1. From a bottle containing mud from Marysville Slough at the low tide mark.
2. From the same bottle 22 days later.
3. From a bottle containing mud from Lake Washington near a sewer outlet.
4. From a bottle containing garden soil from the University campus.

Since it was desired to limit our study to anaerobic or facultative, preferably spore-forming, bacteria which ferment s.w.l. with the evolution of gas, our procedure was as follows: A portion of the sample of liquid taken from a well-shaken bottle was heated for 10 minutes at 85°C., and dilutions of heated and unheated samples were plated in duplicate, using glucose agar. The duplicate plates were incubated at room temperature, one set aerobically and the other in McIntosh anaerobic jars.

The number of bacteria obtained is shown by the following typical example of the counts in the first isolation:

	Bacteria per cc.
Aerobic, heated	1,700
Aerobic, unheated	3,700,000
Anaerobic, heated	79,000
Anaerobic, unheated	48,000,000

From 10 to 20 colonies were selected from each set of plates. It was found that only bacteria picked from anaerobic plates seeded with the heated inoculum produced both acid and gas in glucose and s.w.l. media, whereas all others produced either acid without gas or did not attack carbohydrates at all. Similar results were obtained in all later isolations.

A preliminary study was made, of all gas-forming bacteria isolated to determine their morphology, oxygen requirements and action on gelatin, milk, nitrates and tryptophane. On the basis of these reactions and growth characteristics nineteen strains were selected for detailed pure culture studies.

METHODS AND TECHNIC

The cultures selected for pure culture studies were plated twice, and well-isolated colonies selected.

All bacteria here reported are obligate anaerobes. They were cultivated in McIntosh jars incubated at room temperature.

Although it is customary to use a slightly alkaline medium for the cultivation of anaerobes (Dernby and Blanc, 1921; Kahn, 1922) the organisms studied by us grew better in media with a somewhat acid reaction. All media used in this work, unless otherwise specified, had a pH of 6.8.

It was found that the organisms were particularly sensitive to oxygen when grown in a medium which was sugar-free or contained s.w.l., and that in these cases it was essential to use freshly sterilized (121°C.), rather than merely boiled media.

The organisms grew very scantily in the complete absence of sugars, but as little as 0.01 per cent of glucose produced a markedly stimulating effect. In the presence of sugar the rapidly growing cultures did not sporulate and, unless transplanted within 48 hours, died out. When carried on sugar media with frequent transplanting, the strains became attenuated and eventually died.

Surface growth on any medium (under anaerobic conditions)

could be initiated with great difficulty and then only if sufficient free moisture was present.

The stock cultures were carried in the form of stabs in deep tubes of sugar-free agar, containing 5 grams peptone, 5 grams beef extract, and 15 grams agar per liter, with a pH of 6.8. In this medium only a moderate growth is obtained, most of the cells sporulating in three to four days. These sporulated cultures may be removed from McIntosh jars and stored in an ice-box; they retain their activity for many months, giving vigorous growth on transplanting into a suitable medium.

Inoculation of differential media was made from young (24 to 48 hour) cultures growing on 0.1 per cent glucose agar slants (1.2 per cent agar) which were started from spores. Similar cultures were used for staining characteristics and morphology, and spore stains were made, using cells taken from three or four day-old plain agar slants.

For the study of surface colonies the technic of Reddish and Rettger (1924) was used; the plates were incubated in McIntosh jars for ten days.

Sugar media could not be used for the study of deep colonies owing to the large gas production, which breaks up the agar around the colonies; therefore, nutrient agar (1.0 per cent agar) deep tubes were used. As only microscopic colonies could be obtained when shake cultures were used, a special technic was developed: first, a stab was made and incubated for three days; it was then placed in boiling water for two minutes, the melted agar shaken and a few loopfuls transplanted into a second tube of the same medium (also melted); both tubes were then cooled and incubated. In this way each colony was started from a spore, and depending on the number of spores originally present, either the original or the transplanted tube was found to contain satisfactory colonies. Readings were made after two weeks.

Media for determination of the reactions on milk, gelatin, nitrate, peptone broth, glucose broth, brain media, blood serum, and for hemolysis of blood, were prepared in accordance with accepted methods. Coagulated egg albumin medium was prepared according to Kahn (1922).

Hydrolysis of starch was determined on a medium consisting of nutrient agar with 1.0 per cent of starch. The test was performed in two ways; (*a*) cultures were grown on this medium poured in Petri dishes; the presence or absence of unhydrolyzed starch was demonstrated with iodine after seven days; (*b*) deep tubes of starch agar were stabbed with inoculum; cultures which hydrolyzed starch grew luxuriantly with the production of great quantities of gas; those that did not hydrolyze starch grew only moderately well and did not produce gas. Results obtained by both methods agreed, but the second method is to be preferred for those organisms which fail to grow readily on the surface of agar media.

Lipase production was tested with Berry's butter agar (1933). We have also found that fresh whipping cream in about 5 per cent concentration may be added to agar instead of butter, with the advantage that the fat of the cream remains finely and uniformly distributed in the agar, so that the cream medium can be used either in Petri dishes or as stabs.

Acid and gas production in carbohydrates were determined in broth containing 0.5 per cent of the test substance in the case of common sugars and 0.3 per cent with rare and expensive compounds. In testing the fermentation of organic acids their sodium salts were used. In cases where only a small amount of gas was produced or negative results were obtained in the above carbohydrate-broth media we found that the results could be very satisfactorily and conclusively checked by the use of agar stabs of the following composition: 1.2 per cent agar, 1.0 per cent peptone, and 0.5 per cent of the carbohydrate tested (*without* beef extract). The advantages of this medium are that (*a*) the organisms grow better in agar than in a liquid medium, (*b*) the extent of growth is easily observed along the line of inoculation, and (*c*) even the slightest gas production can be noticed, since the agar confines the gas to the line of growth. The omission of the beef extract was found necessary because these bacteria always produced gas bubbles when grown in deep tubes of standard agar media. By using media containing only (*a*) agar and inorganic salts, (*b*) agar and peptone, (*c*) agar, peptone and

inorganic salts, (*d*) agar and beef extract, it was shown that the beef extract was the source of fermentable material.

Glucose-consuming power was determined by the method of Reddish and Rettger (1924). The residual sugar was determined on sterilized cultures after three, and on duplicates, after seven days of growth. The results given in table 1 show the percentage of sugar consumed as determined by comparison with a blank, which was subjected to identical treatment (including the second autoclaving).

Sulfite waste liquor medium consisted of s.w.l. diluted 2 to 3 with water (40 per cent s.w.l.) and contained 3 grams beef extract and 3 grams peptone per liter. It was always inoculated within a short time after sterilization from a vigorous young culture growing on sugar-free media such as stab cultures in 0.5 per cent peptone, 1.2 per cent beef extract, 1.0 per cent agar, pH 6.8. This parent stab culture was always started from spores.

All cultural reactions were checked with media and cultures prepared on at least two different occasions.

RESULTS

The results of this study show that five distinct species were present. A comparison of their growth characteristics, morphology, physiology and the end products of fermentation with those of previously described bacteria show them to be new species. The following names have been given to them:

1. *Clostridium polyfermenticum, sp. nov.* (nine strains).
2. *Clostridium saccharophilicum, sp. nov.* (three strains).
3. *Clostridium saccharopetum, sp. nov.* (one strain).
4. *Clostridium saccharopostulatum, sp. nov.* (two strains).
5. *Clostridium sartagoformum, sp. nov.* (three strains).

Cl. sartagoformum, sp. nov., is a slender rod, decidedly curved, and forms oval terminal spores. The rod with a spore attached has the appearance of a frying pan with a long curved handle.

The cells of the other four new species resemble each other in appearance, being straight, thick rods producing large, elongated spores which are considerably larger in diameter than the rods. The typical position of these spores is eccentric, the ratio of

length of the shorter part of the rod to the longer part being about as 2 to 3 or as 1 to 2. One may also frequently observe organisms with the shorter end of the rod disintegrated while the other end remains attached to the spore giving the appearance of a terminal spore. However, spores sufficiently mature to give a good spore stain do not long remain attached to the rods.

The absence of proteolytic action and the ability to attack a large number of carbohydrates, as well as the production of butyric acid as one of the end-products, indicates the relation of all five of these species to that large, poorly-described group of anaerobic bacteria usually grouped together under the name of *Clostridium butyricum* (Bergey, 1934, p. 471). However, the new species differ from *Cl. butyricum* in many ways: (*a*) Morphologically, in that slender forms do not occur, spores are eccentric or terminal, and rods are swollen on sporulation; (*b*) in growth characteristics and colony form, both on the surface and in stabs; (*c*) physiologically, in that they do not reduce nitrates, nor fix atmospheric nitrogen; and in that butyric and acetic acids constitute over 98 per cent of the volatile end-products of carbohydrate metabolism under all experimental conditions tried. The organic nitrogenous content was varied over a wide range and incubation was carried out at different temperatures without materially altering this percentage of acid production.

When Bergey's (1934) classification is followed the new species are found to be closely related to *Cl. tertium* (Henry) (1916–17); *Cl. flabelliferum* (Sturges and Reddish) (1926); *Cl. fissum* (Debono) (1912), and *Cl. caloritolerans* (Meyer and Lang) (1926). Since bacteria with eccentric spores are sometimes grouped with the central-spore producing organisms the same key would place the new species near *Cl. multifermentans-tenalbus* (Stoddard) (1919), and *Cl. fallax* (Weinberg and Sequin). However, these bacteria differ from the new species here described in many important characteristics.

Taken as a group the new species are characterized by their unusually diversified and vigorous saccharolytic action and by the complete absence of any proteolytic action. Thus, they do not

liquefy gelatin nor blood serum, produce no indol from peptone, do not hemolyze blood, digest nor blacken brain media nor attack coagulated egg albumin: milk is peptonized by only one of the

TABLE 1

Fermentation reactions of the new species

	CL. POLYFERMENTICUM	CL. SACCHAROPHILICUM	CL. SACCHAROPETUM	CL. SACCHAROPOSTULATUM	CL. SARTAGOFORMUM
Pentose:					
Xylose	X	X	X	X	X
Hexoses:					
Glucose	X	X	X	X	X
Levulose	X	X	X	X	X
Galactose	X	X	X	X	X
Disaccharides:					
Sucrose	X	X	X	X	X
Lactose	X	X	X	X	X
Maltose	X	X	X	X	X
Trisaccharide:					
Raffinose	X	X	X	X	X
Polysaccharides:					
Starch	X	X	X	X	–
Inulin	X	–	–	X	X
Glucoside:					
Salicin	X	X	X	X	X
Alcohols:					
Ethanol	X	–	–	–	–
Glycerol	X	X	–	X	–
Mannitol	X	–	–	–	X
Dulcitol	X	–	–	–	–
Organic acids:					
Acetic	X	X	X	X	X
Butyric	X	X	X	X	X
Glucose consuming power, per cent of the original sugar fermented					
In 3 days	50	41	33	44	43
In 7 days	56	43		57	48

X = acid and gas; – = no fermentation.

species (*Cl. saccharopetum*). However, organic nitrogenous material is essential for their growth and they do not grow in a synthetic medium (containing K_2HPO_4, NH_4NO_3, $MgSO_4$, $CaCl_2$, NaCl and $FeCl_3$); they do not ferment glucose nor s.w.l. in the

absence of an organic source of nitrogen for which peptone, beef extract, asparagine, urea or urine may serve.

Their most outstanding characteristic is the ability to grow in and ferment s.w.l. in dilutions up to 40 to 50 per cent of liquor, when the latter contains some nitrogenous material, e.g. 0.2 per cent beef extract. Butyric and acetic acids and considerable amounts of gas are produced under these conditions.

Morphologically *Cl. polyfermenticum*, *Cl. saccharophilicum*, *Cl. saccharopetum*, and *Cl. saccharopostulatum* resemble each other closely, but differ markedly in this respect from *Cl. sartagoformum*. They are best distinguished from one another on the basis of colony characteristics, and, particularly, on the basis of their fermentation reactions which have been summarized in table 1.

1. Clostridium polyfermenticum

Rods: 0.7 to 0.9 by 2.5 to 3.5 μ, occurring singly and in short chains, ends rounded; actively motile spores subterminal or terminal 1.0 by 1.7 to 2.5 μ. Gram-positive, even in old cultures.

Surface colonies on agar: Vary considerably with the strain. Convex to capitate, discrete, circular, white to creamy in color, opaque, moist, glistening surface, sometimes slightly rough, appear granular in transmitted light, margin slightly irregular; consistency firm and homogeneous with tendency to be sticky; papillae frequently present. Dissociates, which are common, are much thinner and have a cut glass appearance; may form individual colonies or appear around edges of normal colonies. Colony size 1 to 4 mm. in diameter.

Deep colonies in 1 per cent agar: 0.5 to 1 mm., white, compact center with radiating branches; snowflake-like.

Gelatin stab: No liquefaction.

Litmus milk: Acid, coagulated, with some gas formation. Early reduction of litmus.

Peptone broth: Indol not formed, clear.

Glucose broth: Turbid; gas bubbles.

Acid and gas in xylose, glucose, levulose, galactose, sucrose, lactose, maltose, raffinose, starch, inulin, salicin, ethanol, glycerol, mannitol, dulcitol, acetate, propionate, butyrate, sulfite waste liquor.

Blood serum not liquefied; scant growth.

Brain medium not blackened; some gas formed.

Coagulated egg albumin not attacked.

Nitrates not reduced.

Blood not hemolysed.

Potato: Small discrete white colonies. Copious gas formation in surrounding liquid.

Sulfite waste liquor is fermented in 40 per cent concentration with formation of butyric and acetic acids, hydrogen and carbon dioxide.

Optimum temperature 37°C.

Obligate anaerobe.

Habitat: Isolated from mud of brackish waters.

2. Clostridium saccharophilicum

Rods: 0.6 to 0.8 by 2.2 to 4.0 μ, occurring singly, chains very seldom formed; ends rounded; actively motile; spores subterminal 1.1 by 1.8 to 2.1 μ. Gram-positive, even in old cultures.

Surface colonies on agar: Pulvinate or capitate with tendency toward concentric rings, discrete, circular, white, opaque, moist, glistening, edges somewhat uneven, consistency firm, homogeneous and buttery, papillae seldom formed. Size 2 to 4 mm. in diameter.

Deep colonies in 1 per cent agar: Heavy, irregular, compact central mass with a halo of filamentous branching growth.

Gelatin stab: No liquefaction.

Litmus milk: Acid, coagulated, with some gas formation.

Peptone broth: Indol not formed, clear.

Glucose broth: Turbid; gas bubbles.

Acid and gas in xylose, glucose, levulose, galactose, sucrose, lactose, maltose, raffinose, starch, salicin, glycerol, acetate and butyrate, but not in inulin, ethanol, mannitol and dulcitol.

Blood serum not liquefied; scant growth.

Brain medium not blackened; some gas formed.

Coagulated egg albumin not attacked.

Nitrates not reduced.

Blood not hemolysed.

Potato: Raised, discrete, smooth, white, glistening colonies. A large amount of gas formed in surrounding liquid.

Sulfite waste liquor is fermented in 40 per cent concentration with formation of butyric and acetic acids, hydrogen and carbon dioxide.

Optimum temperature 37°C.

Obligate anaerobe.

Habitat: Isolated from mud of brackish waters and from lake-bottom mud polluted with sewage.

3. Clostridium saccharopetum

Rods: 0.8 by 2.5 to 3.5 μ, occurring singly: ends rounded; actively motile; spores subterminal 1.3 by 2.1 μ. Gram-positive, even in old cultures.

Surface colonies on agar: Flat, with raised center, somewhat spreading, white opaque, moist, glistening, edges lobate and rough; sticky in consistency. Surface growth initiated with very great difficulty. Size 3 to 4 mm. in diameter.

Deep colonies in 1 per cent agar: Symmetrical with branching rays radiating from a compact center.

Gelatin stab: No liquefaction.

Litmus milk: Acid, coagulation, followed by slow peptonization; some gas formed.

Peptone broth: Indol not formed, clear.

Glucose broth: Turbid; gas bubbles.

Acid and gas in xylose, glucose, levulose, galactose, sucrose, lactose, maltose, raffinose, starch, salicin, acetate, and butyrate, but not in inulin, ethanol, glycerol, mannitol, or dulcitol.

Blood serum not liquefied; scant growth.

Brain medium not blackened; some gas formed.

Coagulated egg albumin not attacked.

Nitrates not reduced.

Blood not hemolysed.

Potato: Raised, white, smooth, glistening colonies. Gas formed in surrounding liquid.

Sulfite waste liquor is fermented in 40 per cent concentration with formation of butyric and acetic acids, hydrogen and carbon dioxide.

Optimum temperature 37°C.

Obligate anaerobe.

Habitat: Isolated from sewage-polluted lake mud.

4. Clostridium saccharopostulatum

Rods: 0.6 to 0.8 by 2.0 to 4.0 μ, occurring singly; ends rounded; actively motile; spores subterminal 1.0 by 1.8 μ. Gram-positive, even in old cultures.

Surface colonies on agar: Capitate, sometimes convex with depressed edges, discrete, circular, white to cream in color, opaque, surface moist, glistening; consistency firm and homogeneous with tendency to become mucilaginous; edges somewhat uneven. Dissociates have rough, granular surface. Size 1 to 3 mm. in diameter.

Deep colonies in 1 per cent agar: Compact, lumpy, irregular with occasional filamentous growths extending outward.

Gelatin stab: No liquefaction.

Litmus milk: Acid, coagulated, with some gas formation.

Peptone broth: Indol not formed, clear.

Glucose broth: Turbid; gas bubbles.

Acid and gas in xylose, glucose, levulose, galactose, sucrose, lactose, maltose, raffinose, starch, inulin, glycerol, acetate, butyrate, but not in ethanol, mannitol, and dulcitol.

Blood serum not liquefied; scant growth.

Brain medium not blackened; some gas formed.

Coagulated egg albumin not attacked.

Nitrates not reduced.

Blood not hemolysed.

Potato: White, moist, glistening, spreading coalescent growth. A large amount of gas formed in surrounding liquid.

Sulfite waste liquor is fermented in 40 per cent concentration with formation of butyric and acetic acids, hydrogen and carbon dioxide.

Optimum temperature 37°C.

Obligate anaerobe.

Habitat: Isolated from sewage-polluted lake mud.

5. Clostridium sartagoformum

Rods: 0.3 to 0.5 by 3.5 μ, curved; occurring singly; ends rounded; motile; spores terminal 0.8 to 1.0 by 1.1 to 1.5 μ; the sporulated rod has a typical appearance of a "frying pan" with a long curved handle. Gram-positive, even in old cultures.

Surface colonies on agar: Convex, discrete, circular, either white and opaque or transparent dew-drop like; surface moist, glistening and smooth; margin is even, smooth; consistency soft. Size 1 to 2 mm. in diameter.

Deep colonies in 1 per cent agar: 1 to 1.5 mm. in diameter, regular, lens-shaped colonies.

Gelatin stab: No liquefaction.

Litmus milk: Acid, coagulated, with some gas formation.

Peptone broth: Indol not formed, clear.

Glucose broth: Turbid; gas bubbles.

Acid and gas in xylose, glucose, levulose, galactose, sucrose, lactose, maltose, raffinose, inulin, salicin, mannitol, acetate, butyrate, but not in starch, ethanol, glycerol, or dulcitol.

Blood serum not liquefied; scant growth.
Brain medium not blackened; some gas formed.
Coagulated egg albumin not attacked.
Nitrates not reduced.
Blood not hemolysed.
Potato: Very scant growth; no gas in surrounding liquid.
Sulfite waste liquor is fermented in 40 per cent concentration with formation of butyric and acetic acids, hydrogen and carbon dioxide.
Optimum temperature 37°C.
Obligate anaerobe.
Habitat: Isolated from garden soil.

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CHARACTERISTICS OF A NEW SPECIES OF THE GENUS LISTERELLA OBTAINED FROM HUMAN SOURCES

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During the years 1933 and 1934, four individuals died at the New Haven Hospital from an infection, caused by an unusual organism, in which sepsis, focal necrosis of liver and meningitis were the predominant features of the disease. In a separate communication (Burn 1936), detailed clinical and pathological features of the fatal infection caused by this hitherto undescribed species of the genus *Listerella* will be described. The specific organism was isolated either during the clinical course of the disease or postmortem. It was the only organism demonstrable by cultural or staining methods in the lesions of three of the individuals and it was associated with *Pneumococcus type III* in the fourth instance. What proves to be the same organism has been described in the literature; firstly, in a non-fatal illness of an adult (Schultz, Terry, Brice and Gebhardt, 1934) and secondly, from cattle dying with suppurative meningitis (Jones and Little, 1934) (Seastone, 1935). More recently, another strain has been received through the courtesy of Dr. W. Allen of the Hartford General Hospital, Hartford, Connecticut. It was procured from a twenty-six-year-old male dying from meningitis within five to six days after the apparent onset of illness. At present, therefore, there are six known strains of this organism procured from recognized human infections and a similar strain of bovine origin. In view of the clinical and pathological findings in these cases, this new pathogen seems to have a special predilection for localization in the tissues of the central nervous system.

A preliminary report (Burn, 1934) dealt briefly with the cultural and pathogenic properties of this bacterium. This com-

munication purposes to present in greater detail studies of its morphological cultural, serological and pathogenic characteristics.

METHODS

Cultural and biochemical studies

The cultivation of the organism from the blood stream or viscera requires only the usual meat-infusion broth and 5 per cent rabbit or human blood-agar plates as used routinely in any clinical or bacteriological laboratory and adjusted to pH 7.4. Further cultural, and biochemical reactions of the organism are determined with relation to carbohydrate fermentation, action on litmus milk, and gelatin and to the production of indol or nitrites. The sugar fermentation tubes contain 1 per cent carbohydrate prepared in a sugar-free base consisting of Dunham's peptone water plus Andrade's indicator. Fermentation tubes are inoculated with 0.1 cc. of an actively-growing strain of the culture and incubated at 37°C. for seven days. Changes in the fermentation are recorded daily. The final readings are based on observations made with four independent sets of carbohydrate fermentation. The final hydrogen ion concentrations for the strains are determined in 1 per cent glucose broth by means of a glass electrode.

Serological Tests

The vaccine is prepared from a twenty-four-hour washed blood agar plate suspension of the organism killed by heat at 60°C. for one hour. Specific immune rabbit serum is prepared for each strain by administering to full-grown rabbits (weighing 6 to 8 kgm.) on three successive days a gradually increasing concentration of the heat-killed vaccine followed by an interval of rest for three days. After the rest period a second series of inoculations with greater concentrations of vaccine follows until a total of fifteen inoculations have been administered. An interval of two weeks is allowed to elapse before testing the titre of the serum by the agglutination method. The antigen for the test is prepared from twenty-four-hour broth cultures of the organism washed in saline three times before using. The test is incubated

at 37°C. for two hours at 10°C. over night before final readings are recorded. Absorption tests with the method described by Krumwiede, Cooper and Provost (1925) proved satisfactory.

Pathogenicity studies

Young albino rabbits weighing 3 to 4 kgm. were employed in these experiments. The greater portion of these rabbits were raised in this laboratory. They were free from snuffles and only two out of 100 rabbits show histological evidence of rabbit encephalitis as described by McCarty (1924). Guinea pigs used in the experiments weighed 250 to 300 grams. White mice were raised in this laboratory and were free of mouse typhoid. Young *Macacus rhesus* monkeys were retained in quarantine for three weeks before using.

An eighteen to twenty-four-hour meat-infusion blood broth culture was employed for inoculation into animals. Dilutions varying from 10^{-1} to 10^{-7} were prepared in sterile physiological saline and blood agar pour plates were made for the purpose of estimating quantitatively the number of bacilli per cubic centimeter inoculated into the animals. Intravenous inoculations, unless otherwise indicated in the experiment, were never greater in volume than 1 cc. of the particular dilution employed. Dilutions of the twenty-four-hour broth culture ranging from 10^{-1} to 10^{-3} were the quantities found to be optimum for producing infection in the animals. All animals found *in extremis* were sacrificed by means of ether anaesthesia. Necropsies were performed under aseptic conditions and bacteriological cultures obtained from the heart's blood and organs, including the brain and meninges. Sections stained with hematoxylin and eosin were prepared from all the organs except the brain and spinal cord. These latter tissues were stained with toluidine blue by the Nissl method.

Morphological and cultural characteristics

The organism is a non-spore-forming gram-positive bacillus measuring 1 to 4μ in length and 0.5μ in breadth. Occasionally, filaments varying from 5 to 8μ may be observed in some of the

cultures. The organisms usually occur singly or in small clumps of three or four bacilli in an eighteen-hour infusion broth culture, but in recently isolated cultures (fig. 1) they tend to form short chains of three to six bacilli. In older cultures there is a slight tendency to palisade formation, but this certainly is not a characteristic. Irregular, clubbed, barred, segmented and branching forms are not present, but polar-staining bacilli are observed in aged broth cultures. They are readily stained by all the usual dyes. Attempts to demonstrate the presence of a capsule by means of Hiss capsule stain were unsuccessful. There was definite evidence of true motility. Leifson's (1930) flagella stain as modified by Seastone (1935) revealed single polar flagella on all four strains. The organism is readily cultivated on ordinary media without the aid of an additional nutritive substance such as serum or whole blood. It grows as well under reduced oxygen tension as in the presence of oxygen. It grows diffusely in broth in twenty-four hours at 37°C. and remains viable for six months or longer at this temperature. Colony formation on surface blood agar plates in twenty-four hours at 37°C. is characterized by a round, convex, smooth, semi-translucent colony surrounded by a narrow zone of clear hemolysis. Within forty-eight hours the colonies become slightly larger and show very little tendency to change beyond this time of incubation. In pour plates the colonies are biconvex with a clear beta zone of hemolysis (fig. 2). It is difficult to distinguish them from colonies of *Streptococcus hemolyticus*. Hemolysis also occurs in blood broth cultures in twenty-four hours with either rabbit or human red blood cells. Filtrates of forty-eight-hour broth cultures contain a substance capable of hemolyzing washed rabbit blood cells in two hours at 37°C., although the concentration of this substance is slight in comparison with certain strains of *Streptococcus hemolyticus*. All strains of the organism ferment the following carbohydrates without gas formation within twenty-four hours: glucose, maltose, salicin, galactose, dextrin, trehalose, starch and rhamnose. Lactose, glycerol and sorbitol are fermented, but only after a delay of forty-eight hours or more. Mannitol, dulcitol, xylose, levulose, raffinose, inulin and inositol are not changed after fourteen

days' incubation at 37°C. Litmus milk is slowly acidified and finally decolorized, but it is not coagulated. Gelatin is not liquified. Indol is not produced nor are nitrates reduced to nitrites.

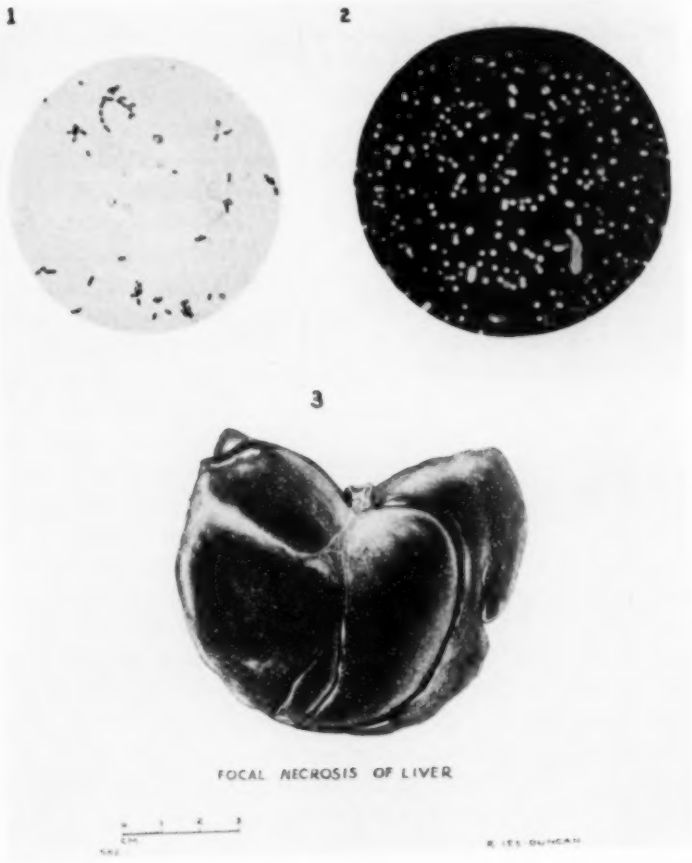

FIG. 1. TWENTY-FOUR-HOUR BLOOD BROTH CULTURE SHOWING CHAIN FORMATION
Strain A3074. ×1000

FIG. 2. DEEP COLONIES IN POUR BLOOD AGAR PLATES
Forty-eight hours at 37°C. Strain A3063

FIG. 3. DRAWING OF RABBIT LIVER SHOWING FOCAL ZONES OF NECROSIS

Serological studies

The results of the agglutination test in serum prepared from all four strains is shown in table 1.

Furthermore, each of the four strains of the organism absorbed completely the antibodies from all four sera.

It may be concluded, therefore, that these four strains are identical in their morphological, cultural and serological characteristics.

TABLE 1

NUMBER OF STRAIN	NUMBER OF IMMUNE SERA																													
	2810								3063								3074								3167					
	DILUTIONS OF SERA																													
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Cont.	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Cont.	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Cont.	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Cont.
2810	C	C	C	C	C	C	C	0	C	C	C	C	C	+	0	0	C	C	C	C	C	+	0	0	C	C	C	C	C	++	0	0
3063	C	C	C	C	C	C	C	0	C	C	C	C	C	C	0	0	C	C	C	C	C	C	C	0	C	C	C	C	C	C	C	0
3074	C	C	C	C	C	C	C	0	C	C	C	C	C	+	0	0	C	C	C	C	C	C	C	0	C	C	C	C	C	C	+	0
3167	C	C	C	C	C	C	C	0	C	C	C	C	C	++	0	0	C	C	C	C	C	C	C	0	C	C	C	C	C	C	C	0

0 = No agglutination; + = slight agglutination, ++ = moderate agglutination; and C = complete agglutination.

Variants

Variants have been obtained from time to time during the course of study either by cultures from organs of animals succumbing to the infection or by successive daily transfer in 10 per cent homologous immune sera. The first recognized variant consists of a strain that has completely lost the property of hemolyzing blood in the agar plates and in broth. Its morphological, cultural and serological properties are not otherwise changed. It was procured in the form of isolated colonies from blood agar plates rich in hemolytic colonies prepared directly from the kidneys of each of four rabbits that died from the infection. It has not been encountered in cultures made from other viscera. Attempts to produce this variant by growing the organism in the presence of sterile rabbit kidney in broth have not, so far, been successful.

Another variant was obtained by successive transfers of the organism to broth containing 10 per cent homologous immune

serum. After ten to twenty transfers, a colony develops that differs from the original in its marked irregularity at the edges after twenty-four hours' incubation at 37°C. and the development of a flat, irregular-edged, translucent spreading growth within forty-eight to seventy-two hours. It still retains, however, the property of hemolyzing blood. Instead of showing the usual regular small rod forms, long filaments measuring 10 to 20 μ in length without branching are observed in stained preparations. This variant retains this characteristic only when kept on solid media, as it reverts to the original type in broth. Sugar fermentations remain unaltered.

The third variant has recently arisen spontaneously from one of the cultures kept without transfer for six to eight weeks in meat infusion broth. It corresponds to the "R" variants that are frequently observed in other strains. The colony is recognized by a wrinkled, dry and irregular edge which tends to be friable upon removal from the plates and by a granular growth in broth. The morphology remains the same, but fermentation of lactose, glycerol and sorbitol within twenty-four hours at 37°C. differs from the delayed fermentation observed in the original strains.

PATHOGENICITY FOR ANIMALS

Rabbits, guinea pigs, mice and monkeys were employed in the study of the pathogenicity of these strains. Since all four strains were capable of producing similar clinical and anatomical changes in animals, the experimental studies have been confined largely to one strain.

Attempts to demonstrate the presence of a toxin in young or old broth cultures were not successful. Large quantities of filtrates had no apparent effect on rabbits, guinea pigs or mice when injected intravenously, intraperitoneally or intracerebrally.

Pathogenicity for rabbits

When quantities of bacilli varying from 100,000 to 10,000,000 per cubic centimeter are inoculated intravenously into rabbits, the following symptoms ensue. The rabbits remain well for

thirty-six to forty-eight hours when they gradually develop anorexia, weakness and signs of central nervous system involvement characterized by twitchings, convulsions, opisthotonas, paralysis of the extremities and finally death within four to five days. Rabbits rarely survived quantities that approximated 10,000,000 bacilli per cubic centimeter. Those receiving a greater dosage died within forty-eight to seventy-two hours from an overwhelming septicemia before localization in the central nervous system was established. Those receiving less than 100,000 per cubic centimeter survived and were sacrificed by the fourteenth day after inoculation. Anatomical lesions at postmortem became more extensive with the length of the time interval for which the animals survived after inoculation. Rabbits receiving quantities of bacteria varying from 100,000 to 10,000,000 per cubic centimeter consistently reveal changes in the liver and brain. The heart, lungs, spleen and adrenals were only occasionally involved. On the other hand, rabbits dying within forty-eight to seventy-two hours showed only lesions in the liver.

The most outstanding gross anatomical change is the diffuse distribution throughout the liver (fig. 3) of small, yellow, focal zones of necrosis. Their extent undoubtedly varies directly with the initial quantity of bacilli inoculated. Microscopically, they (fig. 4) show necrotic liver cells, hemorrhage, infiltration with polymorphonuclear leucocytes and a few small and large mononuclear cells. When lesions are present in other organs, they are similar to the focal necroses of the liver. In the myocardium, however, distinct abscesses were encountered. The spleen and lymph nodes of the rabbit rarely show these lesions, but instead they are congested, with occasional hemorrhage into the splenic tissue.

Rabbits surviving the infection for a period of more than 6 days show reparative processes. At times the proliferation in these foci closely resembles the formation of a tubercle, characterized by an increase of large mononuclear cells which are arranged in a concentric manner. Occasionally, a large multinucleated cell is found in these lesions. Other zones undergo repair by the formation of dense fibrous connective tissue and still others, no doubt,

heal by resolution as there is evidence of hepatic cell regeneration in some foci.

In the gross, the rabbit's brain reveals nothing unusual except in those cases surviving for several days after inoculation, when one observes a gray exudate covering the cortical surfaces and less frequently, the base. Few of the rabbits show areas of softening and necrosis of the cerebral parenchyma, especially in the region of the cerebellum. Subarachnoid hemorrhages also occur in some instances. Histologically, the outstanding pathological picture is the extensive meningitis (fig. 5) that extends into the sulci and fissures of the cortex and by way of the perivascular sheaths into the cerebral parenchyma. This exudate is comprised primarily of polymorphonuclear leucocytes with scattered large mononuclear cells. All of the microscopic preparations reveal a marked angeitis of the pial vessels with swelling of the endothelial cells to cause almost complete obliteration of their lumina. Swelling and necrosis of the ganglion cells are observed in some of the sections. Subarachnoid hemorrhages are seen microscopically in 20 per cent of the rabbits examined. In those sacrificed after the fifth day, large mononuclear cells predominate, with an occasional polymorphonuclear leucocyte in the exudate. Glial proliferation is observed in some of the sections.

If the organism is inoculated into the subarachnoid space via the basal cistern, it requires only 10 to 30,000 bacilli to produce clinical evidence of marked meningeal irritation, characterized by convulsions and opisthotonas which is followed by death in twenty-four to forty-eight hours. At postmortem, an extensive suppurative meningitis is always present in these rabbits.

On the other hand, when animals are inoculated other than by the intravenous or intracerebral routes, the number of bacilli necessary to produce infection is either so large that it causes death within forty-eight to seventy-two hours without anatomical evidence of localization or the host survives without showing anatomical changes when it is sacrificed later. Both the intraperitoneal and subcutaneous routes of inoculation require quantities of bacilli varying between 1,000,000 and 1,000,000,000 per cubic centimeter in order to produce infection which results

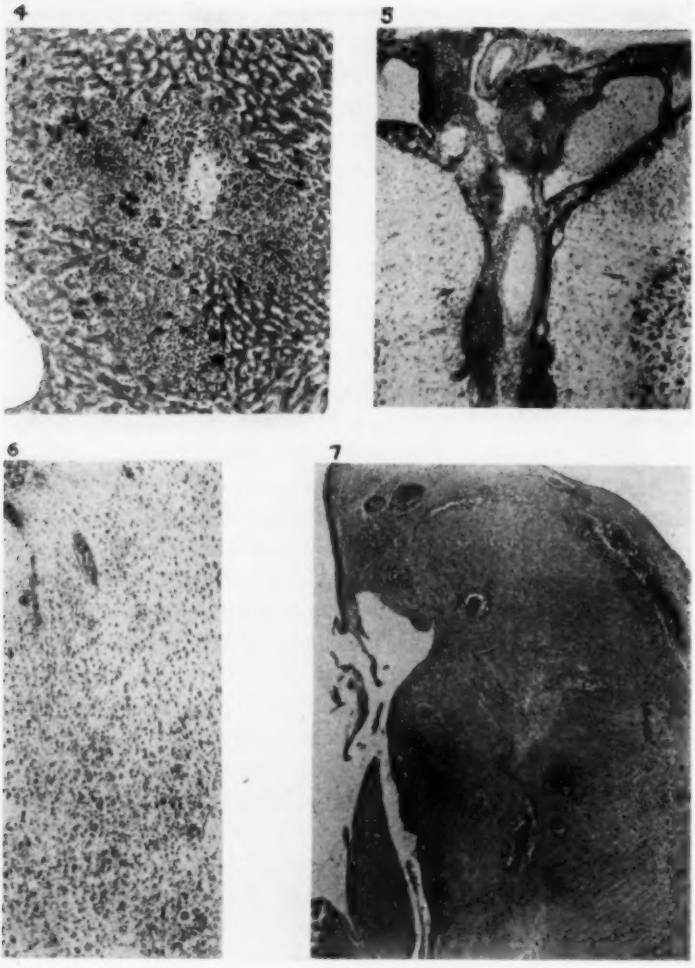

FIG. 4. MICROSCOPIC ZONES OF FOCAL NECROSIS IN LIVER OF RABBIT. ×70

FIG. 5. DIFFUSE MENINGITIS AND ANGEITIS IN CEREBRUM OF RABBIT. ×70

FIG. 6. DIFFUSE MENINGO-ENCEPHALITIS IN CEREBRUM OF MONKEY

Swelling and necrosis of ganglion cells. Polymorphonuclear leucocytic infiltration throughout cortex. MVIII. ×70.

FIG. 7. MULTIPLE ABSCESSES IN MYOCARDIUM OF GUINEA PIG. ×30

in death in forty-eight to seventy-two hours without any evidence of central nervous system involvement either clinically or at postmortem. A suspension of the organism placed in the nares produced infection in one out of five rabbits. This animal showed a pulmonary abscess from which the organism was isolated in pure culture. It is significant that an extensive suppurative meningitis was also present.

The organism is demonstrable by cultural and staining methods in the lesions as well as in the viscera showing no anatomical changes. It is important to note that a higher incidence of positive cultures is obtained from the viscera of these animals than from the heart's blood. Such cultures were even obtained from the viscera of some of the rabbits that were sacrificed from six to fourteen days after inoculation. Specific agglutination titer of the sera of the rabbit that survived the infection varied from 1:20 to 1:100 dilution of sera. However, observations pertaining to the immunological responses of these rabbits must await further study.

Table 2 summarizes the clinical, pathological and bacteriological findings of the rabbits inoculated by the different routes.

Pathogenicity for Guinea Pigs

Intravenous inoculation of the organism in equivalent quantities elicits an entirely different response in the guinea pig than in the rabbit. Quantities of 100,000 to 60,000,000 bacilli per cubic centimeter cause the guinea pig to develop signs of weakness, labored breathing and inactivity. Death occurs after 5 days or more. Clinical evidence of involvement of the central nervous system is not present. At postmortem, foci of necrosis similar to those seen in the rabbit are observed in the liver. The guinea pig differs from the rabbit inasmuch as the myocardium (fig. 6) is invariably the seat of multiple abscesses. A fibrinous pericarditis was present in three of the animals and a mural thrombus of the left ventricle occurred in another.

On the other hand, involvement of the central nervous system of guinea pigs is brought about by inoculating the organism either intracerebrally or intraperitoneally. It requires only minimum

TABLE 2

Rabbits

NUMBER OF BACILLI INOCULATED	TOTAL NUMBER OF RABBITS	DIED 1 TO 5 DAYS	SURVIVED 6 TO 14 DAYS	CLINICAL SYMPTOMS	PATHOLOGY IN ORGANS			BACTERIOLOGICAL FINDINGS		
					Liver	Brain	Other organs	H.B.	Liver	Brain
Intravenous										
								1 cc. volume		
6,000–100,000	7	3		3	2	3	0	0	3	3
			4	0	1	1	0	0	1	1
100,000–1,000,000	5	3		3	3	3	Heart—1	1	3	3
		1	2	0	0	0	0	0	0	0
1,000,000–10,000,000	16	9		8	9	9	Heart—2	5	9	8
			7	3	0	3	Spleen—1	0	1	2
10,000,000–1,000,000,000	4	4		4	3	4	Adrenal—1	3	4	4
			0	0	0	0	0	0	0	0
Total	32	19		18	17	19	Heart—3	9	19	18
			13	3	1	4	Spleen—1 Adrenal—1	0	2	3
Intraperitoneal										
100,000–1,000,000	1		1	0	0	0	0	0	0	0
1,000,000–10,000,000	2	1		1	1	0	Peritonit.—1	1	1	1
			1	0	0	0	0	0	0	0
10,000,000–1,000,000,000	5	4		4	4	0	Peritonit.—3	4	4	4
		1	1	0	0	0	0	0	0	0
Total	8	5		5	5	0	Peritonit.—4	5	5	5
		1	3	0	0	0	0	0	0	0
Intracerebral										
3,000	1	1		1	0	1	0	1	1	1
3,000,000	1	1		1	0	1	0	1	1	1
Total	2	2		2	0	2	0	2	2	2

TABLE 3

Guinea pigs

NUMBER OF BACILLI INOCULATED	TOTAL NUMBER OF ANIMALS	DIED 1 TO 5 DAYS	SURVIVED 6 TO 14 DAYS	CLINICAL SYMPTOMS	PATHOLOGY IN ORGANS			BACTERIOLOGICAL FINDINGS		
					Liver	Brain	Other organs	H. B.	Liver	Brain
Intravenous										
								1 cc. volume		
100,000–1,000,000	2	0	2	0	1	0	Heart—2 Pericard.—1	0	1	0
1,000,000–60,000,000	4	4	0	4	4	0	Heart—4 Pericard.—2 Adrenal—1 Kidney—1	4	4	4
Total	6	4	2	4	5	0	Heart—6 Pericardi.—3	4	5	4
Intraperitoneal										
40,000–100,000	1	0	1	1	0	0	Heart—0 Spleen—1 Adrenal—1	0	1	1
100,000–1,000,000	2	0	2	2	1	1	0	1	1	1
1,000,000–10,000,000	1	0	1	1	1	1	0	1	1	1
Total	4	0	4	4	2	2	Spleen—1 Adrenal—1	2	3	3
Intracerebral										
								0.2–0.3 cc. volume		
2,000–100,000	5	4	1	4	1	4	0	1	3	4
		1		0	0	0	0	0	0	0

quantities to produce infection when the organisms are inoculated into the subarachnoid space via the basal cisterns. The one failure to produce meningitis by this mode of inoculation was probably because the material did not enter the subarachnoid

space. Larger quantities of organisms are required to produce infection by the intraperitoneal route. However, only 50 per cent of the guinea pigs develop definite clinical symptoms referable to the central nervous system and show histological evidence of a meningitis.

These animals survived for a period of six to fourteen days and finally expired in convulsions. The myocardium of these animals

TABLE 4

Mice

NUMBER OF BACILLI INOCULATED	TOTAL NUMBER OF MICE	DIED 1 TO 5 DAYS	SURVIVED 6 TO 14 DAYS	CLINICAL SYMPTOMS	PATHOLOGY IN ORGANS			BACTERIOLOGICAL FINDINGS		
					Liver	Brain	Other organs	H.B.	Liver	Brain
Intraperitoneal										
								1 cc. volume		
60,000–100,000	24	8		8	8	0	Spleen—8	8	8	8
			16	0	0	0	Heart—1	0	0	0
100,000–1,000,000	12	12		12	12	1	Spleen—12	12	12	12
			0							
1,000,000–25,000,000	19	19		19	19	0	Spleen—12	19	19	19
			0							
Total.....	55	39		39	39	1	Spleen—39	39	39	39
			16				Heart—1			
Intracerebral										
								0.3–0.5 cc. volume		
100–100,000	6	6		6	0	6	0	6	6	6

showed no lesions. The remaining guinea pigs survived and showed no evidence of infection either pathologically or bacteriologically.

Pathogenicity for mice

Mice develop a suppurative meningitis within twenty-four to forty-eight hours after the inoculation of small quantities of

bacilli into the brain tissue. Intraperitoneal inoculations of relatively small numbers of bacilli produce a septicemia and death within forty-eight hours. At postmortem, a diffuse fibrinous peritonitis and multiple foci of necrosis in the liver and spleen are the only anatomical changes. The presence of necrotic zones in the spleen of mice is the outstanding anatomical lesion since it is not observed in the organs when rabbits or guinea pigs are the hosts. Table 4 summarizes the findings in white mice.

Pathogenicity for monkeys

Four monkeys (*Macacus rhesus*) inoculated intravenously with two or three times the number of bacilli inoculated into the rabbits develop clinical signs or symptoms that directly point to involvement of the central nervous system. The onset of the clinical symptoms usually occurs between the second and fifth day after inoculation, characterized by anorexia, ruffling of hair, twitchings of muscles, clashing and grinding of teeth, photophobia, weakness and marked rigidity of the neck. The first monkey recovered from the symptoms; two were sacrificed immediately before expiring, the fourth when evidence of recovery was apparent.

Examples of two of the protocols are given for monkeys III and VIII.

Monkey III.

5/4/34. Temperature 102°F. Inoculated into the femoral vein 3 cc. of a twenty-four-hour blood broth culture. Total 1,000,000,000 bacilli.

5/5/34. Temperature 102.5°F. Inactive; loss of appetite.

5/6/34. Temperature 104°F. Inactive; marked weakness; chattering of teeth. Rigidity of neck. Blood culture—no growth.

5/7/34. 9:00 a.m. Temperature 104.5°F. Extremely weak. Breathing rapidly and with difficulty. Twitching and tremor of muscles. Loss of balance. Nystagmus. Blood culture—no growth.

5/7/34. 3:00 p.m. Temperature 105°F. Semi-comatose. Breathing very difficult. Marked arching of spine. Sacrificed by ether anaesthesia.

Necropsy. There is no gross or microscopic evidences of anatomical changes in the lungs, liver, spleen or kidneys. Upon removal of the calvarium, the dura appears tense and gray. The blood vessels over the cortical surfaces are markedly engorged, but gross evidence of exudate is only recognized at the base of the brain. Histologically, there is an extensive exudate characterized by polymorphonuclear cells which are located around the pial vessels and extend by way of the perivascular spaces into the deeper portions of the brain parenchyma. The sulci and fissures of the cortex of the brain show accumulations of polymorphonuclear cells. Ganglion cells (fig. 6) appear swollen and necrosis of some of these cells occurs, in addition to a diffuse infiltration of polymorphonuclear cells throughout the brain parenchyma.

Postmortem bacteriology. The heart's blood at postmortem remained sterile but the viscera, including lungs, liver, spleen and kidneys revealed the organism. However, cultures of the meninges and brain tissue were heavily positive for the organism, as indicated by numerous colonies on the direct surface blood agar plates. The agglutination test of the animals' sera failed to show antibodies.

Monkey VIII.

5/11/34. Temperature 101°F. Inoculated into the femoral vein 1 cc. of a twenty-four-hour blood broth culture. 2,500,000 bacilli per cubic centimeter.

5/12/34. Temperature 103.2°F. Inactive and ruffled hair. Sudden twitchings and jerking of head.

5/13/34. Temperature 103°F. Very weak and inactive. Twitchings of head and muscles increased. No appetite.

5/14/34. Same general condition. Unable to orient itself when removed from cage. Blood culture—no growth.

5/15/34. Temperature 103°F. Stronger although very weak. Twitchings much less.

5/16/34. Temperature 102°F. Much improved. Twitchings absent. Recognition of surroundings. Blood culture—no growth. Sacrificed by ether anaesthesia.

Necropsy. There are no gross or microscopic changes found in the viscera. Gross examination of the brain shows nothing unusual. Histologically, there is a perivascular infiltration in the meninges consisting of mononuclear and occasional polymorphonuclear cells. No changes are observed in the parenchyma of the brain.

Postmortem bacteriology. The culture of the heart's blood at post-

mortem showed no evidence of growth. The spleen was the only organ, aside from the meninges and brain tissue, from which the organism could be cultivated. Serological examination of the serum shows the presence of agglutinins at a dilution of 1:40.

The first monkey (MI) which recovered completely showed no pathological or bacteriological evidence of the infection ten days after the initial inoculation. The fourth monkey (MV) revealed similar anatomical changes to those observed for monkey III.

DISCUSSION

One of the most interesting properties of this new species of the genus *Listerella*, disclosed by these studies, is its unusual ability to pass from the blood stream into the central nervous system of rabbits and monkeys. Other kinds of organisms isolated from human brain tissue at necropsy do not tend to localize in the central nervous system of animals when inoculated in small quantities into the venous system or even into the carotid arteries. It is possible, however, to produce an occasional instance of central nervous system involvement in experimental animals by inoculating massive quantities of an avirulent organism into the circulation or by using a suspension of organisms and media that favors the passage of infected emboli to the blood vessels of the brain. Since it is difficult to reproduce, consistently, experimental meningitis in animals by way of the blood stream, it has been hypothecated that an actual barrier exists between the circulation and the cerebral tissue. Therefore, this new organism may serve as an experimental means for studying the mechanism of the invasion into the central nervous system from the blood stream.

Some of the factors governing localization in the central nervous system are suggested by these experiments. The time of entrance of organisms into the central nervous system as manifested clinically and pathologically occurs about the third to fifth day after inoculation. In order to show this time relationship, it is essential to inoculate into the nervous system numbers of bacteria, ranging between 100,000 to 10,000,000 bacilli, as a larger number of bacilli cause early death of the animal, while smaller quantities

usually fail to produce infection. However, study of the histological preparations of rabbits sacrificed at the initial onset of the central nervous system involvement shows that the predominating lesion consists of swelling of the endothelial cells of the smaller blood vessels and a marked angeitis. Therefore, it is possible that this vascular injury may be an essential preliminary for the invasion of the organism into the subarachnoid space.

Other unrecognized factors, no doubt, exist in influencing the localization of this organism as is shown by the differences in the lesions presented by guinea pigs. Intravenous inoculations of small quantities of bacilli produce multiple myocardial abscesses without any evidence of central nervous system involvement. On the other hand, 50 per cent of the guinea pigs inoculated by the intraperitoneal route did develop clinical and pathological evidence of meningitis without showing lesions in the myocardium. The factors controlling this unusual localization remain for further investigation.

It is difficult to classify this organism properly at this time. It has been included among the diphtheroids or genus *Corynebacterium* on the basis of morphological studies but other results based on cultural and serological experiments do not substantiate this grouping. Studies have suggested that it is closely allied to an organism described by Murray, Webb and Swanson (1926) that is known now by the name of *Listerella monocytogenes* (1934). A more detailed and systematic comparative study of several related organisms is now in progress and will be reported in another communication.

SUMMARY

1. The organism described in this communication is a small non-spore forming gram-positive bacillus that has a tendency to form short chains and small clumps in broth. A clear beta zone of hemolysis is produced in blood agar plates. Hemolysis of blood in meat infusion broth also occurs in twenty-four hours at 37°C.

2. Cultural and morphological characteristics indicate that this organism is probably a new species of the genus *Listerella*.

3. Specific agglutinin and absorption tests show that the four strains of this new pathogen are identical.

4. It is pathogenic for rabbits, guinea pigs, mice, monkeys and man.

5. Lesions produced by the organism consist primarily of a focal necrotizing process and exudation which are most marked in the liver.

6. Localization of the organism in the central nervous system of rabbits and monkeys results when it is inoculated into the venous blood system. On the other hand, intravenous inoculations into guinea pigs cause multiple myocardial abscesses instead of meningitis.

7. Intracerebral inoculations of very small quantities of this organism directly into the subarachnoid space invariably produce an extensive suppurative meningitis in rabbits, guinea pigs, mice and monkeys.

8. Intraperitoneal inoculations into guinea pigs cause localization of the organism in the central nervous system without evidence of myocardial involvement. This route of inoculation for rabbits and mice proves fatal in 48 hours if optimum dosages are employed.

9. *Macacus rhesus* monkeys show a remarkable degree of resistance, surviving the infection even after it has once become established within the central nervous system.

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THE RELATION OF OXIDATION-REDUCTION POTENTIAL TO THE GROWTH OF AN AEROBIC MICROÖRGANISM[1]

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INTRODUCTION

The possible relationship between the oxidation-reduction potentials of culture media and the ability of microörganisms to initiate growth has recently interested bacteriologists. The work of Aubel and Aubertin (1927), Dubos (1929a), Fildes (1929), Knight and Fildes (1930), Plotz and Geloso (1930), and others, has established the fact that the growth of certain anaerobes is greatly influenced by the oxidation-reduction potential of the medium. In the case of aerobic bacteria, however, conflicting results have been reported. Allyn and Baldwin (1930, 1932) demonstrated that the potential of the medium was of considerable importance in determining whether or not small inocula of *Rhizobium*, an aerobic organism, could initiate growth. Knaysi and Dutky (1934), on the other hand, finding that *Bacillus megatherium* would not grow in the absence of dissolved oxygen, even when the potential of the medium was varied over a considerable range, concluded that "the limiting factor in the growth of *Bacillus megatherium* in vacuum is the oxygen content and not the oxidation-reduction potential of the culture medium." Their experiments offer no information, however, as to whether or not the potential of the medium affects the growth of this organism under *aerobic conditions*.

The purpose of this paper is to report: (*a*) experimental evi-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

dence indicating that the growth of an aerobe (*Bacillus megatherium*) in the presence of adequate oxygen is influenced by changes in the oxidation-reduction conditions of the medium, and (*b*) roughly quantitative data as to the range of potential in which such an aerobic organism most readily initiates growth.

EXPERIMENTAL

The oxidation-reduction conditions in the culture medium were varied in two ways: first, by the addition of "poising agents" in the form of reversible oxidation-reduction systems, such as the indicator dyes, which alter the capacity of the medium at definite potential zones; second, by the addition of K_2SO_3 which reduces the medium and so changes its potential.

Freshly prepared broth adjusted to a pH of 7.2 ($\pm$0.1) was used in all experiments unless otherwise stated. Its composition was as follows:

Beef extract	3 grams
Peptone	5 grams
K_2HPO_4	2 grams
Distilled water	1000 cc.

The indicators used were manufactured by the La Motte Chemical Products Company, with the exception of phenosafranine, neutral red, and methylene blue, which were purchased from the National Aniline and Chemical Company, Inc. The indicators were prepared in sterile water under aseptic conditions immediately before use. The aerobe studied was *Bacillus megatherium*; all cultures were incubated at 37°C.

I. The effect of oxidation-reduction indicators on growth

Dubos (1929b) studied the effect of a group of oxidation-reduction indicators upon the growth of several facultative anaerobes, and found that the dyes with E_0' values above and including that of methylene blue, when in the oxidized form, were toxic to pneumococci and hemolytic streptococci of human and bovine origin. On the other hand, when reduced, the dyes were no longer toxic.[2]

[2] Cohen, Chambers, et al. (1929) noted the relative non-toxicity of these dyes in the reduced form when injected into marine ova.

The dyes having E_0' values below that of methylene blue showed no bacteriostatic action, with the exception of phenosafranine, neutral red, and Janus green, which were shown to have bactericidal properties other than those which could be attributed to their effect upon oxidation-reduction conditions, and were therefore not included in the final data. It should be emphasized that since the indicators are added in small concentrations in the oxidized form, they do not change the potential of the medium appreciably. The effect is rather to add "capacity" to the me-

TABLE 1

Action of reversible indicators upon the growth of B. megatherium in broth culture

COMPOUND	E_0' (VOLT) AT pH 7.2 AND 30°C.	GROWTH IN 24 HOURS
o-Chlorophenol indophenol	+0.218	−
Phenol indophenol	+0.212	−
o-Cresol indophenol	+0.180	−
1-Naphthol-*2*-sulfonate indophenol	+0.111	−
Thionine	+0.056	−
Methylene blue	+0.004	−
Indigo tetrasulfonate	−0.055	+
Indigo trisulfonate	−0.091	+
Indigo disulfonate	−0.134	+
Indigo monosulfonate	−0.165	+

The indicators were added to tubes of broth to give a final concentration of 1.8×10^{-4} molar. The tubes were then inoculated with 0.1 cc. of a twenty-four-hour broth culture of *B. megatherium* diluted 1:10, and observed for growth after twenty-four hours' incubation at 37°C.

dium at a definite zone of potential (dependent upon the particular dye), and thus to stabilize the potential at this level when the medium is being reduced by the organism. His results led Dubos to offer the hypothesis that "the 'inhibiting' dyes 'poise' the medium at a potential outside the range in which the inhibited organism can grow."

A technique similar to that described by Dubos was used in testing the effect of oxidation-reduction indicators upon the growth of *Bacillus megatherium*. The indicators used are listed in table 1 in the order of the electromotive series. The action of

phenosafranine and neutral red was also studied, but these tests were not included in the final data for reasons similar to those mentioned by Dubos. The dyes were added to 5 cc. portions of medium to give final concentrations of from 2×10^{-6} to 1.8×10^{-4} molar, and the tubes were inoculated with 0.1 cc. of a twenty-four-hour broth culture at dilutions of 1:10, 1:100, and 1:1000. After incubation for twenty-four hours, the tubes were examined for growth. Representative results of duplicate experiments with the highest concentration of dye and the largest inoculum have been recorded in table 1. When smaller concentrations of the dyes were used, it was found that the larger inocula were able to overcome the bacteriostatic effect of the "inhibiting" dyes. In the case of these latter, additional tests were made, in which sufficient quantities of K_2SO_3 were added to the dye-medium mixture to reduce the dye to the colorless form. None of the dyes inhibited growth when so reduced. The results obtained with *Bacillus megatherium* were thus strikingly in accord with those of Dubos with the facultative anaerobes.

In the hope of obtaining further evidence as to the nature of the inhibitory action of the dyes, experiments were carried out to determine (*a*) the effect of the dyes on the growth curve, and (*b*) whether or not the dyes, in the concentrations used, actually poised the medium to any appreciable extent.

II. Time-potential relationships

Allyn and Baldwin in 1930 pointed out the importance of oxidation-reduction potential in determining the length of the lag phase of *Rhizobium*. Ingraham (1933) concluded that gentian violet owes its bacteriostatic action, at least in part, to its ability to poise the potential of the medium and showed that it affects growth only during the lag phase. If the inhibitory oxidation-reduction indicators act through a similar mechanism, it should be possible, by using suitable concentrations of the "inhibiting" dye, to demonstrate an increase in the length of the lag phase.

Due to the difficulty of making accurate direct counts on *Bacillus megatherium* because of its tendency to grow in chains and to form clumps, an indirect electrometric technique was used

in studying the length of the lag phase. The method employed was based upon the common observation (Frazier and Whittier, 1931) that the beginning of the logarithmic phase of growth practically coincides with a sharp drop in the potential of the medium. This fact was confirmed in the case of *Bacillus megatherium* in broth culture by simultaneous potentiometric measurements and microscopic counts. Although the counts were made with some difficulty, the results were sufficiently uniform to show clearly that the first swift drop in potential coincided with the beginning of the logarithmic phase of growth. Certain of the dyes which were shown earlier to inhibit growth were added to the culture medium to make final concentrations of from 2×10^{-5} to 1.6×10^{-4} molar. Each 5 cc. of medium then received an inoculum of 0.1 cc. of a twenty-four-hour broth culture diluted 1:10. Potential measurements were made at intervals over a seventy-hour period of incubation. The potentiometric set-up was similar to that described by Allyn and Baldwin in 1932. All potentials are referred to the hydrogen electrode. Time-potential curves for inoculated medium containing different amounts of *1*-naphthol-*2*-sulfonate indophenol are shown in figure 1. Similar results were obtained with methylene blue.

The time-potential curves show that increasing concentrations of an "inhibiting" dye tend to lengthen the lag phase. That the dye, in the concentrations used, is effective only during the lag phase may be demonstrated by adding the dye to the medium at various intervals following inoculation. Inhibition of growth occurs only in those tubes receiving the dye before the onset of the logarithmic phase of growth.

The question may be raised as to whether the dyes have been added in sufficient concentration to have any appreciable poising effect on the medium. The time-potential curves of figure 1 show that in the two cases where sufficient dye was added to lengthen the lag phase markedly, a distinct "plateau" was encountered in the region of +0.100 volt. Since the E_0' of the dye lies in this range of potential, it is evident that the "plateau" is caused by the poising action of the dye. The interpretation is substantiated by the fact that the length of the plateau in each

curve is roughly proportional to the dye concentration. Similar curves were obtained by Cannan, Cohen, and Clark (1928) when potentials were followed on a culture of yeast to which methylene blue had been added.

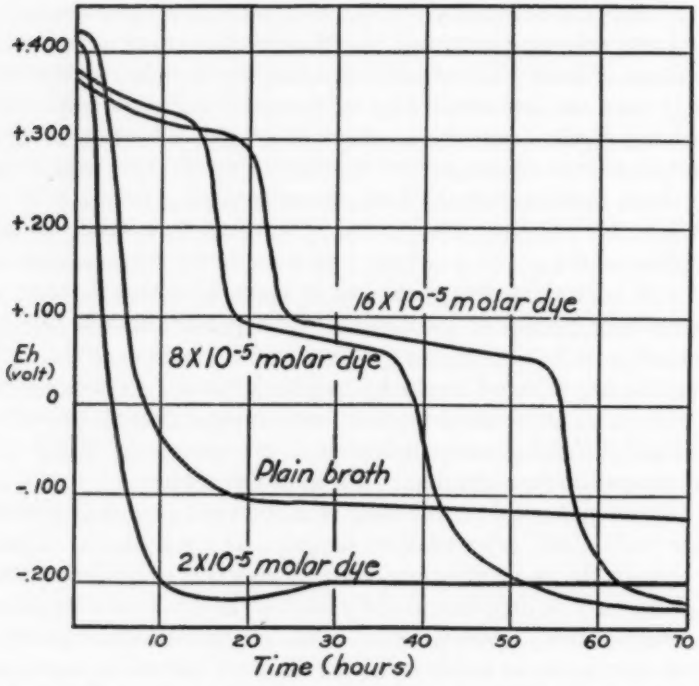

FIG. 1. TIME-POTENTIAL CURVES FOR B. MEGATHERIUM CULTURES IN PLAIN BROTH, AND IN BROTH CONTAINING VARIOUS CONCENTRATIONS OF 1-NAPHTHOL-2-SULFONATE INDOPHENOL

III. Zone of optimum potential for growth initiation

The above experiments indicate that, in general, only those dyes which poise the potential of the medium above a certain definite level inhibit the growth of *Bacillus megatherium*. The relation of the *initial* potential of the medium to the ability of the organism to initiate growth was then investigated.

An inorganic reducing agent, K_2SO_3, was added in varying quantities to obtain media of different potentials. It was found potentiometrically that the potentials of sterile medium thus obtained covered a range of from +0.450 volt to −0.050. Slight jarring of tubes containing a highly reduced medium (the surface of which was not protected from the air) was observed to cause a marked rise in the potential, due undoubtedly to the entrance of oxygen. In all subsequent work, suitable precautions were taken to avoid unnecessary jarring of the tubes. Potentiometric measurements showed that a considerable period of time was required for the sulfite to reduce the medium to a stable potential. In some cases stable potentials were not attained for nearly twenty-four hours. Once a state of equilibrium was reached, a relatively constant potential was maintained for well over twenty-four hours, provided the tubes were carefully handled.

Experiments were carried out in which a series of broth tubes was prepared, to which sulfite had been added to make final concentrations of from 0.003 to 0.03 per cent.[3] The tubes were allowed to stand for forty-eight hours at 37°C. so that stable potentials might be reached. They were then inoculated and the time of first appearance of turbidity was recorded. The differences observed with heavy inocula were too slight to be conclusive, and the results obtained with highly diluted inocula lacked uniformity, probably because of clumping. However, inoculation with a twelve-hour broth culture diluted 1:1000 to 1:10,000 gave reproducible results. Representative readings are shown in figure 2, and indicate that broth containing from 0.015 to 0.021 per cent K_2SO_3 is considerably more suitable for growth initiation than is the untreated broth. The potential of the medium exhibiting the most favorable conditions was determined both potentiometrically and colorimetrically, and was found to be roughly between 0.00 and −0.05 volt. This observation becomes significant when it is recalled that of all the dyes studied, only those which tend to poise the medium at potentials above 0.00 volt inhibited growth.

[3] In preliminary experiments no growth appeared within twenty-four hours in tubes containing more than 0.025% sulfite.

Confirmation of this work using other reducing agents is of course to be desired, and should be sought in subsequent investigations.

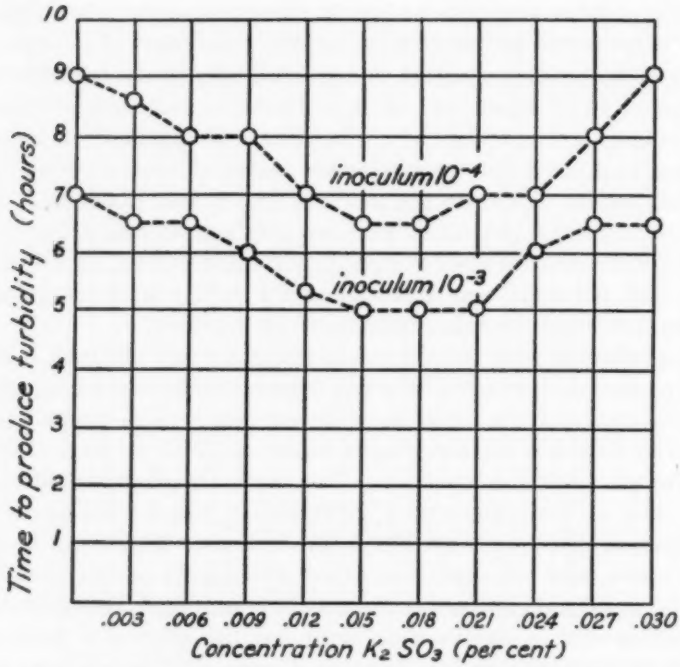

FIG. 2. THE EFFECT OF POTASSIUM SULFITE IN BROTH ON GROWTH INITIATION BY B. MEGATHERIUM

K_2SO_3 was added to a series of broth tubes in the amounts indicated, and the tubes were allowed to stand without agitation for forty-eight hours. They were then inoculated with a diluted broth culture of *B. megatherium* and observed for first appearance of turbidity.

DISCUSSION

An interesting working hypothesis has been suggested to explain the manner in which oxidation-reduction conditions affect bacterial suspensions. Evidence has been amassed by various investigators that bacteria can multiply only in media having a potential below a fairly definite "critical" value. The well-known

fact that bacteria can lower the potential of common media suggests that an organism ordinarily lowers the potential of the medium in its immediate vicinity to the "critical" value before starting to multiply. The addition of a reagent which oxidizes the medium, or an oxidation-reduction system which can poise it above the "critical" potential zone, would be expected to make it more difficult for the organism to reduce the medium locally, and therefore cause a certain degree of bacteriostasis, which will depend upon the concentration of the oxidizing or poising agent, and the size and degree of dispersion of the inoculum (Dubos, 1929a, b; Allyn and Baldwin, 1930). Conversely, if the medium has been reduced to a potential below the "critical" value at the time of inoculation, the organism may begin to multiply almost immediately, and the lag phase may be appreciably shortened (Allyn and Baldwin, 1932). The ability of the organism to initiate growth will thus depend upon the relation of the oxidation-reduction conditions of the medium to the "critical" potential.

Whether or not this hypothesis is ultimately proved to be correct, the evidence afforded on the one hand by the indicator experiments and on the other by the sulfite results here reported seems to support this line of reasoning. In conclusion, the data presented indicate that the growth of *Bacillus megatherium* in the presence of oxygen sufficient for growth is definitely influenced by the oxidation-reduction conditions of the culture medium.

SUMMARY

1. Reversible oxidation-reduction indicators positive to, and including, methylene blue inhibit the growth of *Bacillus megatherium* when in the oxidized form. Indicators negative to methylene blue in the electromotive series fail to inhibit growth. None of the compounds which inhibit growth in the oxidized form are bacterio-static when reduced.

2. Potentiometric measurements indicate (*a*) that the bacterio-static compounds studied affect growth only during the lag phase, and (*b*) that in these experiments sufficient indicator was added to the medium to have an appreciable poising effect. Such observations support the hypothesis suggested by Dubos that

the bacterio-static action of these compounds is a function of their ability to poise the potential of the medium.

3. Beef-extract broth to which suitable amounts of an inorganic reducing agent has been added, is more favorable for growth initiation by *Bacillus megatherium* than untreated broth. The potential range of the medium in which growth is most readily initiated is in the neighborhood of 0.00 to −0.05 volt.

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SOME QUANTITATIVE RELATIONSHIPS IN BACTERIAL POPULATION CYCLES[1,2]

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Although bacterial population cycles have been rather extensively studied qualitatively, accurate quantitative interpretation of certain aspects of the growth phases is wanting. This is due chiefly to the lack of comparable data on various organisms obtained under controlled, reproducible environmental conditions.

The present investigation was undertaken to define more precisely the reproductive cycles of some of the common saprophytes under controlled, comparable conditions. In particular, the effect of temperature on reproduction was studied. From these growth curves, certain generalized quantitative relationships are derived.

No attempt is made here to review as a whole the literature on growth curves; this has been done adequately by Henrici (1928), Buchanan and Fulmer (1928, 1930), and Rahn (1932). A complete review is also given in the author's original thesis (Jennison, 1932).

METHODS

Broadly, the experiments involved growing several organisms in broth at various temperatures under accurately controlled

[1] Portion of a thesis submitted to the Graduate School of the Massachusetts Institute of Technology, May, 1932, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

[2] Contribution No. 57 from the Department of Biology and Public Health, Massachusetts Institute of Technology.

conditions, and determining their growth curves over a twenty-four-hour period by plate count.

The species of bacteria used were a secondary consideration except insofar as they fulfilled certain desiderata of technical procedure, namely, ease of handling, absence of spores, and relatively little tendency of the cells to remain attached to each other after reproduction. The following organisms were chosen as representative of four genera of common saprophytes: *Escherichia coli*, an old stock culture and a newly isolated strain; and old stock cultures of *Aerobacter aerogenes*, *Serratia marcescens*, and *Chromobacterium violaceum*. Purity of the cultures was ascertained by the usual methods.

The organisms were "acclimatized" to 22°C. by growing on agar slants, with weekly transplants, for four months before use; they were then grown in broth, with transfers every twelve hours, for a week at this temperature. Growth curves were determined at 22°, 27°, 32°, 37°, and 42°C.—except for the last two species which did not reproduce at 42°—using the 22° cultures for inoculation. (Each organism was also acclimatized (as above) to 27°, 32°, 37°, and, except for the two species mentioned, 42°C. The growth curve of each organism was then determined at the temperature to which it had been acclimatized, by the same procedures as given below. With one exception, the growth of each organism at a given temperature after acclimatization to that temperature was quantitatively the same (within the limits of experimental error) as when acclimatized to 22°. The data for this second series of experiments are not included in this paper, since they were so nearly identical with those reported. The exception mentioned will be discussed later.)

For each organism the growth curves were determined at the four or five temperatures at the same time. Series of culture tubes containing 9 ml. of nutrient broth, which had been brought up to temperature, were inoculated by means of pipettes delivering a known number of drops per milliliter, with 1 to 3 drops of a twelve-hour broth culture of the particular organism, thus keeping the volume of medium approximately constant. As determined by previous experiment, this inoculum gave 1000 to

2000 cells per milliliter, the exact number being found by plate count each time. This quantity is large enough to prevent irregularities in the growth curve, and small enough to give a long period of reproduction. The cultures were immediately incubated at the various temperatures.

At exactly two-hour intervals for twelve hours, and in many cases for twenty-four hours, two (new) tubes were removed at each temperature, stoppered with sterile rubber stoppers, shaken for one minute, and the contents pooled and mixed. Serial dilutions were made in 9 ml. water blanks at room temperature and immediately (within five minutes) plated, 5 plates being made from the proper dilutions. The use of 5 plates for obtaining averages gives more reliable results than does a lesser number (Wilson, 1922; Wilson and Kullmann, 1931). Furthermore, as Wilson and Kullmann have shown statistically, if three or more plates are made in parallel, the elimination of any one plate that is markedly different from its replicates can be made without bias. In our experiments, replicate plates nearly always checked within a few per cent; those few differing by more than 20 per cent (four times the average deviation) from the average were omitted.

Plates were inverted and incubated for forty-eight hours at 37°C. before counting. The dilutions giving 100 to 400 colonies per plate were used for enumeration, which range Wilson (1922) has shown to be optimum for reliable results when averaging three or more plates of the same dilution. Most of our mean counts are averages of five plates.

Conditons of incubation, dilution, etc., were controlled so as to minimize experimental errors. Temperatures of incubation did not vary more than ±0.15°C. from those specified. The same lot of Difco dehydrated nutrient broth was used throughout, being weighed out rapidly on an analytical balance to the nearest tenth of a milligram to give an 0.8 per cent solution in distilled water, and autoclaved at 15 pounds for twenty minutes. All tubes of broth, and water blanks, contained 9.0 ml. ±0.1 ml. of liquid when used. The same lot of Difco dehydrated nutrient agar was also used throughout, and the plates poured at 42.0°C.

±1°C. Dilution pipettes (1 ml.) were selected for uniformity of delivery, the average variation being ±1 per cent of volume.

RELIABILITY OF RESULTS

It is difficult to evaluate properly all of the sources of error involved in growth studies and arrive at an exact measure of the precision of results. The *counting error* (i.e., the measure of the reliability of the mean of several replicate plates of the same dilution) is the one most commonly employed by bacteriologists, and can readily be calculated. It may be expressed most simply as the percentage average deviation of a set of plates from the arithmetic mean, although more precise statistical measures are available (Fisher, Thornton, and MacKenzie, 1922; Fisher, 1930; Wilson and Kullmann, 1931). The *counting error* is *not necessarily* a good measure of the *total* error, since it does not take into account errors of dilution. In this study these latter were so much smaller than the counting error, however, that for practical purposes the counting error could be used as a fairly reliable measure of the precision of results.

In the experiments here reported, involving several hundred sets of plate counts, the counting error was consistently low, the average deviation of individual plates of a set from the mean averaging 5 per cent of the mean. This is equivalent to a standard error of the *means* of about ±3 per cent.

In these studies it was also desirable to determine the precision of the bacterial generation times during the logarithmic growth period. This was accomplished in most cases by averaging the generation times calculated for the (usually) two or more two-hour sampling periods during the logarithmic growth phase, and obtaining the average deviation from the resultant mean. (While this method is not statistically precise it is simple, and the results obtained are, within the limits of experimental error, comparable to the values obtained by using the method of least squares.) In those few cases where the phase of logarithmic growth lasted through only about one sampling period (two hours) this method could not be employed. Instead, a formula (Goodwin, 1920) was used which allows computation of the precision of a result

(in this case, generation time) when the precision measures of the components (plate counts at the beginning and end of the sampling period) are known. It was found that the average deviation of the individual generation times from the means was about 7 per cent of the mean. (Standard error of *means* = ±4 per cent.) Similarly, computation of the precision of the temperature quotients, $Q_{10°}$, gave an average deviation of about 10 per cent. (Standard error = ±6 per cent).

THE GROWTH CURVES

In figures 1 to 5 are shown the growth curves at various temperatures for the five organisms, the data for which curves are in tables A to E (appendix). The curves for the different organisms are comparable, since the inoculum was practically the same in each case. Numbers of cells being plotted logarithmically, the slopes of the curves show relative rates of reproduction directly.

The growth curves as a whole are typical of bacterial population cycles, showing a lag period, a phase of maximum (logarithmic) rate of reproduction, and a long period of decreasing rate of growth (Buchanan, 1918). It will be noted (see also tables A to E, appendix) that particularly at the higher temperatures the beginning and end of the logarithmic growth phase is very sharp, which largely obviates the necessity for subjective judgment in determining the length of this phase.

The presence of a lag period at the various temperatures for all organisms except the old strain of *Escherihia coli*, may be explained, except at 22°, as due to the fact that 22° cultures used for inoculation had to become adapted to the higher temperatures before reproducing maximally, since physiologically young cells are more susceptible to environmental changes than old cells (Sherman and Albus, 1923, 1924; Jensen, 1928). It is not clear why the old *Escherichia coli* showed no lag under these conditions. The presence of a lag period at 22°C. is somewhat unexpected, in view of the results of Chesney (1916). He found with the pneumococcus, *Escherichia coli*, *Pseudomonas fluorescens* and *Serratia marcescens* growing at *optimum temperature* that if the

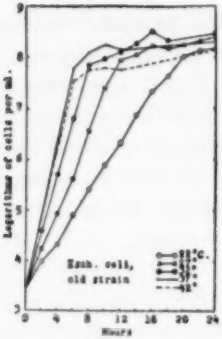

FIG. 1

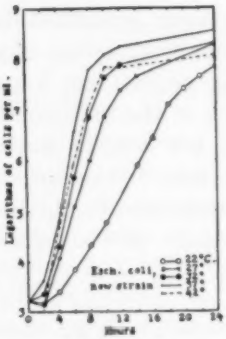

FIG. 2

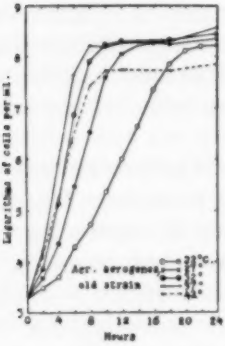

FIG. 3

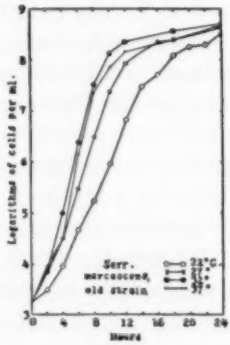

FIG. 4

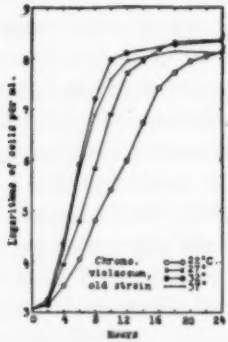

FIG. 5

FIGS. 1 TO 5. GROWTH CURVES OF BACTERIA IN BROTH AT VARIOUS TEMPERATURES, BY PLATE COUNT

culture is reproducing at its most rapid rate (logarithmic growth phase) the bacteria, upon subculture at the same temperature, show no lag period. In our work, the 22°, twelve-hour cultures used for inoculation, while not growing at the optimum temperature, were in the logarithmic growth phase, and it might be expected that upon subculture at 22° the organisms would continue to reproduce maximally. This however, is definitely not the case; the curves for all organisms (with the possible exception of *Escherichia coli*, old strain, which is somewhat irregular during the first four hours at this temperature) show a lag period of from 2 to 4 hours or longer. Duplicate experiments with the five organisms yielded the same results, except that the old strain of *Escherichia coli* showed a definite lag at 22°C. This seems to indicate that the presence of a lag period in subcultures at the same temperature, with bacteria growing at temperatures below their optimum, is dependent, in part at least, upon factors other than the age of the parent culture. Possibly the temperature or the number of cells inoculated is concerned.

Form of the growth curve

There has developed a tendency by some workers to regard bacterial growth in culture as analogous to the autocatalytic phenomena observed in chemistry (Buchanan and Fulmer, 1928, p. 52). To this living phenomenon the name autocatakinetic growth has been applied. A plot of cells against time is represented to be a regular symmetrical sigmoid curve, on which the point of inflection (that point at which the average rate of increase per cell begins to decrease, i.e., the end of the logarithmic phase) is at $b = \frac{a}{2}$, where b is the number of cells at the point of inflection, and a is the maximum bacterial count. While the growth curve of a bacterial culture would be expected to be sigmoid, there is reason to believe that it would not ordinarily be symmetrical about its point of inflection, although there is little experimental data to support adequately either contention. Of two such studies, McKendrick and Pai (1911) found the growth curve of *Escherichia coli* in broth to be reasonably symmetrical;

while Buchanan and Fulmer (1930, p. 63) analyzing the data of Müller on five organisms at various temperatures in a liquid medium, found the growth curves to be exceedingly asymmetrical.

TABLE 1

Point of inflection of growth curves

ORGANISM	TEMPERATURE	"MAXIMUM" NUMBER OF ORGANISMS *a*	NUMBER OF ORGANISMS AT POINT OF INFLECTION *b*	POINT OF INFLECTION IN TERMS OF *a* *a/b*
	°C.	*millions per ml.*	*millions per ml.*	
Esch. coli, old	22°	147.0	20.1	a/7
	27°	246.0	24.4	a/10
	32°	282.0	72.0	a/4
	37°	195.0	60.0	a/3
	42°	136.0	34.3	a/4
Esch. coli, new	22°	68.0	10.2	a/7
	27°	189.0	7.6	a/25
	32°	199.0	7.1	a/28
	37°	350.0	60.0	a/6
	42°	115.0	10.2	a/11
Aer. aerogenes, old	22°	450.0	72.0	a/6
	27°	580.0	42.0	a/14
	32°	670.0	80.0	a/8
	37°	650.0	44.0	a/15
	42°	199.0	27.0	a/7
Serr. marcescens, old	22°	310.0	29.0	a/11
	27°	460.0	23.5	a/20
	32°	490.0	32.0	a/15
	37°	410.0	22.4	a/18
Chromo. violaceum, old	22°	132.0	25.0	a/5
	27°	226.0	50.0	a/4
	32°	216.0	15.7	a/14
	37°	137.0	7.6	a/18

The exact degree of symmetry of our growth curves about the point of inflection, that is, the relative position of this point, could not be determined precisely or directly, without extrapolation from the data, because in no case had the maximum

number of cells been reached. However, by using the number of cells at the end of twenty-four hours as a "maximum" a good approximation was obtained, since such a procedure showed that all the curves were asymmetric in one direction about the point of inflection. Therefore the true figures for total crop would merely show the curves to be more asymmetric in the same direction, and would define more precisely the degree of asymmetry.

In table 1 is given the relative position of the point of inflection of the growth curve of each organism, calculated from the values of $\frac{a}{b}$, where a is the "maximum" number of cells (after twenty-four hours) and b the number of organisms at the point of inflection (end of the logarithmic growth phase). (Data in tables A to E, appendix.) The relative position of each point is given in terms of a, in order to facilitate comparison with the value $a/2$ of a symmetrical curve.

It is apparent that since a symmetrical curve would have its point of inflection at $a/2$, these growth curves are far from symmetrical even when low "maximum" figures are used, the point of inflection in all cases being reached long before it would be for a symmetrical curve. Similar results were obtained in the experiments with acclimatized cultures, all curves being asymmetric. It is impossible to determine directly from our data whether temperature affects the point of inflection, because the true maximum numbers were not reached.

EFFECT OF TEMPERATURE UPON THE MINIMUM GENERATION TIME

The usual numerical measure of the rate of reproduction of bacteria is the generation time (GT), defined as the time (in minutes) necessary for the cells to double in number, assuming that all are dividing. This can be calculated from the well-known formula (Buchanan and Fulmer, 1928, p. 19):

$$GT = \frac{t \log 2}{\log b - \log a}$$

in which t is the time interval in minutes, a is the number of cells at the beginning, i.e., when $t = 0$, and b is the number of cells

after time t. For comparative purposes the minimum generation times (during the logarithmic growth phase) are used.

Generation times were calculated by the above formula from the plate counts made at each two-hour sampling interval (tables A to E, appendix). From these values and an inspection of the growth curves the limits of the phase of logarithmic growth were obtained fairly exactly, since, particularly at higher temperatures, the break at the beginning and end was marked. From the generation times for each two-hour interval during logarithmic reproduction a mean value was obtained. These mean generation times for all organisms are given in table 2. The mean values are calculated only to the nearest minute, since, as we

TABLE 2

Effect of temperature upon the mean generation time during period of logarithmic growth

ORGANISM	MEAN GENERATION TIME IN MINUTES				
	22°C.	27°C.	32°C.	37°C.	42°C.
Esch. coli, old	73	46	33	25	26
Esch. coli, new	65	39	26	20	26
Aer. aerogenes, old	62	33	21	21	29
Serr. marcescens, old	52	37	26	22	
Chromo. violaceum, old	55	34	23	25	

have noted, their standard error is $\pm 4\%$ (about ± 1 minute at 37°C.).

The effect of temperature upon the mean generation time is shown graphically for all organisms in figures 6 and 7.

It was noted previously (p. 2) that in a second series of experiments (unreported) the growth curve (and therefore the mean generation time during logarithmic growth) of each organism at a given temperature after acclimatization to that temperature was, with one exception, the same as when acclimatized to 22°. The exception was *Serratia marcescens* at 37°. The mean generation time during logarithmic growth at this temperature, using the 22° culture for inoculation, was 22 minutes (table 2); the culture which had been acclimatized to 37° for

four months showed a mean generation time at 37° of thirty-three minutes. It is not known how long it took the organism to reach this lower rate, but the observation is of interest in that it brings out the possibility that 37° as an "optimum" for *reproduction* of this organism (table 2 and figure 7) may be more apparent than

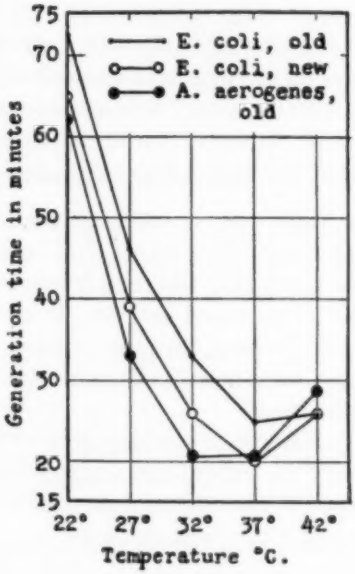

FIG. 6

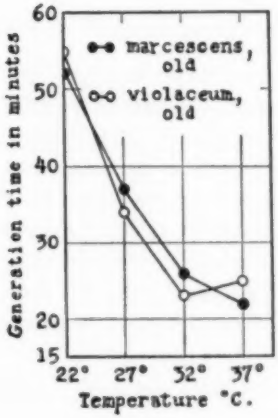

FIG. 7

FIG. 6. EFFECT OF TEMPERATURE UPON THE GENERATION TIME OF BACTERIA DURING THE LOGARITHMIC GROWTH PHASE

FIG. 7. EFFECT OF TEMPERATURE UPON THE GENERATION TIME OF BACTERIA DURING THE LOGARITHMIC GROWTH PHASE

real, depending upon the previous temperature of cultivation of the culture and the phenomenon of "growth momentum."

The faster rate of reproduction at all temperatures up to 42°, of the freshly isolated strain of *Eschichia coli* compared with the old stock culture (figure 6), is worthy of note, and in particular the sharper optimum at 37°. Compared with the two strains of *Escherichia coli*, *Aerobacter aerogenes* seems to have a slightly lower

optimum temperature; its relatively faster rate of reproduction below 37° is also evidence of this. The optimum temperature for *Chromobacterium violaceum* is definitely nearer 32° than 37° (figure 7).

The observed generation times for *Escherichia coli* and *Aerobacter aerogenes* are similar to the values found by other workers (Mason, 1934). The value of twenty-two minutes at 37° for *Serratia marcescens* is considerably smaller than the minimum of thirty-seven minutes recorded for this organism in milk by Heinemann and Glenn (1908). The data for *Chromobacterium violaceum* appear to be new.

THE TEMPERATURE QUOTIENT, $Q_{10°}$, OF THE RATE OF GROWTH

In many chemical reactions a 10°C. rise in temperature increases the rate of reaction two or three times (van't Hoff rule) Since many biological reactions (including reproduction) are governed in part at least by rates of chemical reaction, it is of interest to determine the relationship between the rates of growth of bacteria at different temperatures, even though the significance of the relationship is not well understood.

The ratio between the velocity constants of the rate of growth at two different temperatures may be called the temperature quotient (or coefficient). Customarily, the temperature quotient is recorded for a 10°C. interval as $Q_{10°}$, the formula being

$$Q_{10°} = \frac{k_2}{k_1}$$

in which k_2 is the velocity constant at the higher temperature and k_1 the velocity constant at the lower (Buchanan and Fulmer, 1930, p. 34). The velocity constant k can be calculated from the generation time, as follows (Buchanan and Fulmer, 1928, p. 20):

$$k = \frac{ln\ 2}{GT}$$

It follows from this that

$$Q_{10°} = \frac{GT_1}{GT_2}$$

in which GT_1 and GT_2 are the generation times at the lower and higher temperatures, respectively.

The temperature quotients $Q_{10°}$, for all organisms were calculated by these formulas from the mean generation times during the period of logarithmic growth (table 2) at each temperature. The results are shown in table 3.

As would be expected, the values of $Q_{10°}$ within a particular temperature range vary slightly for different organisms, although the similarities in value are more marked than the differences. It will be noted that $Q_{10°}$ varies inversely with temperature. The temperature quotients are consistently larger in the lower temperatures ranges investigated, so that while with increasing

TABLE 3

Effect of temperature upon the temperature quotient $Q_{10°}$

ORGANISM	$Q_{10°}$ FOR TEMPERATURE INTERVALS		
	22°-32°C.	27°-37°C.	32°-42°C.
Esch. coli, old	2.2	1.8	1.3
Esch. coli, new	2.5	2.0	1.0
Aer. aerogenes, old	2.9	1.6	0.7
Serr. marcescens, old	2.0	1.7	
Chromo. violaceum, old	2.4	1.4	

temperature up to the optimum a concomitant increase occurs in the *absolute* rate of reproduction, it is apparent that the effect of a given temperature increase near the optimum is less, relative to the effect produced by the same increase in the lower ranges. Only within a short range would a 10°C. rise in temperature exactly double the reproductive rate; above this range the effect is less, below it, greater, although the van't Hoff rule may be said to hold roughly.

The order of magnitude of our temperature coefficients is similar to the values observed in the few other cases reported in the same temperature range for bacteria (Barber, 1908; Lane-Claypon, 1909; Chick, 1912) and for yeast (Slator, 1906; Sherwood and Fulmer, 1926).

RELATION BETWEEN THE MAXIMUM RATE OF REPRODUCTION AND LENGTH OF THE LOGARITHMIC PHASE AT THE SAME TEMPERATURE

Inspection of the growth curves (figures 1 to 5) reveals the well-known fact that at lower temperatures the logarithmic phase continues longer, and the rate of reproduction is less, than at higher temperatures. In other words, the duration of the maxi-

TABLE 4

Relation between mean generation time during logarithmic phase and length of logarithmic phase at the same temperature

ORGANISM	TEMPERATURE	MEAN GENERATION TIME (*GT*)	APPROXIMATE LENGTH OF LOGARITHMIC PHASE (*L*)	RATIO (*GT*/*L*)
	°C.	*minutes*	*hours*	
Esch. coli, old	22°	73	12	6.0
	27°	46	10	4.6
	32°	33	8	4.1
	37°	25	6	4.2
	42°	26	6	4.3
Esch. coli, new	22°	65	14	4.6
	27°	39	8	4.9
	32°	26	4	6.5
	37°	20	6	3.3
	42°	26	4	6.5
Aer. aerogenes, old	22°	62	10	6.2
	27°	33	6	5.5
	32°	21	6	3.5
	37°	21	4	5.2
	42°	29	4	7.2
Serr. marcescens, old	22°	52	10	5.2
	27°	37	6	6.1
	32°	26	4	6.5
	37°	22	4	5.5
Chromo. violaceum, old	22°	55	14	4.0
	27°	34	6	5.5
	32°	23	4	5.7
	37°	25	4	6.2

mum rate of reproduction appears to be inversely proportional to that rate. We have found a roughly quantitative ratio between these two quantities, which is about the same at each temperature over the range of these experiments. This ratio for each organism at each temperature is shown in table 4 (data from tables A to E, appendix, and table 2).

In spite of the fact that the duration of the logarithmic growth phase was not exactly determinable in every case, the ratio of generation time (in minutes) to length of logarithmic phase (in hours) is constant, within relatively narrow limits, for the same organism at different temperatures, approximating 5:1 as an average. There seems to be some indication that the ratio changes above the optimum temperature. It may also be seen from table 4 that for different organisms at the same temperature, the ratio is about the same in each case. The experiments with the cultures acclimatized to each temperature yielded almost identical results.

It is recognized, of course, that both the length of the logarithmic phase and the rate of growth therein are dependent upon the size of the inoculum (McKendrick and Pai, 1911; Graham-Smith, 1920), which fact lends weight to the assumption that the constant ratio found between minimum generation time and duration of that time is of general application.

DISCUSSION

The results of the experiments reported here have been presented for the various genera as a group, in order to bring out the general nature of the relationships. There are, of course, variations between the individual genera, although these differences are less marked than the similarities.

The observations in regard to the presence of a lag period at 22° with all organisms, when the inoculum was from cultures reproducing at a maximum rate at that temperature, needs further investigation, since it has been accepted as a general principle—at least for bacteria at *optimum* temperatures—that if the organisms are in the logarithmic growth phase, a subculture at the same temperature will show no lag (Chesney, 1916).

Buchanan and Fulmer (1928, p. 35), note that it is perhaps necessary also to take into account the effect of differences in numbers seeded in generalizing this principle. Apparently this does not hold in the present case, however, because our inoculum was 1000 to 2000 cells per milliliter, while Penfold (1914) found no lag in subculture at 37°C.—using organisms reproducing at a maximum rate—with about 500 cells per milliliter of *Escherichia coli*, and Chesney (1916) got no lag with 80 cells of *Escherichia coli* under similar conditions. Although the true explanation of our lag is not clear, it may be that bacteria *below* their optimum temperature, when subcultured while reproducing maximally, actually do not continue the maximum rate under the same conditions as do bacteria at optimum temperature.

Our results show definitely that bacterial growth curves, although sigmoid, are not symmetrical about their point of inflection. There seems then to be little justification for trying to compare a growth curve to a (symmetrical) curve of chemical autocatalysis. Furthermore, as stated by Kavanagh and Richards (1934), the demonstration of a good fit of such an autocatalytic curve to a given growth curve is no proof that the growth is autocatalytic.

The finding that the apparent optimum temperature for reproduction of *Serratia marcescens* was dependent upon the previous temperature of cultivation is not in itself new as a general principle; Zikes (1919) for example, showed that the growth-rate temperature-curve for yeast is modified by the temperature at which the strain has previously been cultivated. The observation brings up the question, however, as to how an optimum temperature should be determined—whether the growth-rate temperature-curves should be made from cultures carried at "room temperature" or from organisms first acclimatized to the various temperatures. As Stuart (1925) has shown, from a previously favorable environment (in our case, 22°) an organism may gain momentum which permits it to flourish for a time in unfavorable conditions (37°); several transplants in the new environment are necessary before the effect of growth momentum can be ruled out. In most of the experiments here,

acclimatization had no appreciable effect. In the case of *Serratia marcescens*, however, the decrease of eleven minutes in the generation time at 37°C., after cultivation at this temperature for four months, is significant. Zikes (1919, 1920) found that after cultivation of his yeast at low temperature (8°C.) for some time, growth was more rapid at the lower temperatures than before, and that there was a tendency to develop two optima.

The data presented for the temperature quotients, $Q_{10°}$, of the rate of growth at various temperatures, do not cover a wide enough range to draw broad conclusions as to the operation of the van't Hoff temperature rule in bacterial reproduction. Within the range studied, however, there is a definite decrease in the value of $Q_{10°}$ at higher temperatures with all organisms. This finding is in accord with that of most other workers, that the temperature quotient for the reproduction of microörganisms varies inversely with temperature. While it is apparent that qualitatively the effects of temperature upon bacterial reproduction are in some respects analogous to those exhibited in simple chemical reactions, microörganisms, being complex, may follow a "law" of their own.

SUMMARY

Data are presented on the form of the growth curve, and the effects of temperature upon rate of reproduction, as determined from representatives of four genera of common saprophytic bacteria. The growth curves of the organisms, *Escherichia coli*, *Aerobacter aerogenes*, *Serratia marcescens*, and *Chromobacterium violaceum*, were determined at 22°, 27°, 32°, 37°, and 42°C.—enumeration of cells being by plate count. The following results were obtained.

1. Curves of bacterial population cycles are sigmoid, but are not symmetrical about their point of inflection.

2. The presence or absence of a lag period in subcultures at the same temperature, with bacteria whose optimum is about 37°C., but which are being cultivated at 22°C., seems to be dependent, other conditions being equal, upon factors other than the age of the inoculum.

3. With one exception, the maximum rate of reproduction at a given temperature was the same whether the cultures used for inoculation were first acclimatized to that temperature or to room temperature (22°C.). The exception (*Serratia marcescens* at 37°C.) shows that sometimes the previous temperature of cultivation may influence the optimum temperature, at least for reproduction.

4. The minimum generation time of *Serratia marcescens* may be as low as 22 minutes at 37°C.; of *Chromobacterium violaceum*, 23 minutes at 32°C.

5. The *relative* effect of temperature upon maximum rate of reproduction was progressively less from 22° to 42°C., i.e., Q_{10} varies inversely with temperature.

6. At any temperature up to the optimum, the ratio of minimum generation time (in minutes) to length of logarithmic growth period (in hours) at the same temperature was approximately constant (5:1), both for the same organism at different temperatures and for different organisms at the same temperature.

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APPENDIX

TABLE A

Mean plate counts and generation times of Esch. coli, old culture, in broth

AGE OF CULTURE, HOURS	BACTERIA PER MILLILITER, THOUSANDS					GENERATION TIME, MINUTES				
	22°	27°	32°	37°	42°C.	22°	27°	32°	37°	42°C.
0	2.7	2.7	2.7	2.7	2.7					
2	9.5	17.9	40.0	77.0	74.0	66	44*	31*	31	25*
4	20.8	86.0	500.0	2,350.0	1,760.0	106	53*	31*	24*	26*
6	78.0	420.0	5,870.0	60,000.0	34,300.0	63*	53*	35*	26*	28*
8	251.0	3,500.0	72,000.0	126,000.0	56,000.0	71*	39*	33*	112	169
10	720.0	24,400.0	95,000.0	173,000.0	63,000.0	79*	43*	300	227	706
12	1,980.0	90,000.0	132,000.0	145,000.0	57,000.0	83*	64	324	−19	−19
14	6,900.0	117,000.0	183,000.0			67*	316	324		
16	20,100.0	170,000.0	324,000.0			78*	226	146		
18	43,000.0	152,000.0	207,000.0	165,000.0		110	−19	−20	2000	
20	100,000.0	176,000.0				99	571			
22	129,000.0	204,000.0				333	571			
24	147,000.0	246,000.0	282,000.0	195,000.0	136,000.0	667	444	818	1500	576

* Generation time during logarithmic growth period.

TABLE B

Mean plate counts and generation times of Esch. coli, new culture, in broth

AGE OF CULTURE, HOURS	BACTERIA PER MILLILITER, THOUSANDS					GENERATION TIME, MINUTES				
	22°	27°	32°	37°	42°C.	22°	27°	32°	37°	42°C.
0	1.6	1.6	1.6	1.6	1.6					
2	1.5	1.4	2.4	4.2	1.3	−18	−19	194	85	−19
4	2.5	10.2	19.9	62.0	49.0	160	42*	39	31	23*
6	7.1	128.0	470.0	3,700.0	1,010.0	81	33*	26*	20*	28*
8	21.0	1,020.0	7,100.0	60,000.0	10,200.0	77	40*	31	30	36
10	59.0	7,600.0	43,000.0	121,000.0	67,000.0	81	42*	47	119	44
12		23,500.0	78,000.0	173,000.0	66,000.0		74	141	235	−18
14	800.0	44,000.0				64*	133			
16	2,720.0	59,000.0				68*	286			
18	10,200.0					63*				
20	25,400.0					92				
22	43,800.0					154				
24	68,000.0	189,000.0	199,000.0	350,000.0	115,000.0	190	287	534	713	900

* Generation time during logarithmic growth period.

TABLE C

Mean plate counts and generation times of Aer. aerogenes, old culture, in broth

AGE OF CULTURE, HOURS	BACTERIA PER MILLILITER, THOUSANDS					GENERATION TIME, MINUTES				
	22°	27°	32°	37°	42°C.	22°	27°	32°	37°	42°C.
0	1.9	1.9	1.9	1.9	1.9					
2	3.1	4.7	7.6	16.7	10.3	170	92	60	38	49
4	5.1	22.7	146.0	820.0	170.0	170	53	28	21*	30*
6	19.1	268.0	7,500.0	44,000.0	3,200.0	63*	34*	21*	21*	28*
8	53.0	3,400.0	80,000.0	161,000.0	27,000.0	81*	33*	35	64	39
10	228.0	42,000.0	183,000.0	152,000.0	52,000.0	57*	33*	101	−18	128
12	990.0	113,000.0	193,000.0	180,000.0	55,000.0	57*	85	1714	500	1500
14	4,300.0	164,000.0				57*	226			
16	22,700.0	187,000.0				50*	667			
18	72,000.0	175,000.0	207,000.0	193,000.0	52,000.0	72*	−18	3600	1200	−55
20	127,000.0					148				
22	157,000.0					400				
24	165,000.0	218,000.0	280,000.0	370,000.0	70,000.0	1714	1161	837	387	857

* Generation time during logarithmic growth period.

TABLE D

Mean plate counts and generation times of Serr. marcescens, old culture, in broth

AGE OF CULTURE, HOURS	BACTERIA PER MILLILITER, THOUSANDS				GENERATION TIME, MINUTES			
	22°	27°	32°	37°C.	22°	27°	32°	37°C.
0	1.8	1.8	1.8	1.8				
2	3.1	6.8	7.3	8.7	148	62	59	52
4	8.9	33.0	102.0	31.0	79	53	32	80
6	47.0	312.0	2,390.0	1,390.0	50*	37*	26*	22*
8	176.0	3,000.0	32,000.0	22,400.0	63*	37*	32	30
10	900.0	23,500.0	133,000.0	68,000.0	51*	40	59	75
12	6,500.0	85,000.0	217,000.0	143,000.0	42*	65	169	112
14	29,000.0	143,000.0			56*	160		
16	49,000.0	211,000.0			160	214		
18	122,000.0	240,000.0	360,000.0	235,000.0	92	667	493	507
20	176,000.0				231			
22	190,000.0				1091			
24	310,000.0	460,000.0	490,000.0	410,000.0	169	387	818	450

* Generation time during logarithmic growth period.

TABLE E

Mean plate counts and generation times of Chromo. violaceum, old culture, in broth

AGE OF CULTURE, HOURS	BACTERIA PER MILLILITER, THOUSANDS				GENERATION TIME, MINUTES			
	22°	27°	32°	37°C.	22°	27°	32°	37°C.
0	1.2	1.2	1.2	1.2				
2	1.4	1.5	1.8	1.5	462	203	197	353
4	3.4	9.9	21.7	17.4	92	45	33	34
6	11.4	61.0	780.0	520.0	69	46	23*	25*
8	65.0	690.0	15,700.0	7,600.0	48*	34*	28	31
10	262.0	8,000.0	92,000.0	38,000.0	60*	34*	47	52
12	980.0	50,000.0	126,000.0	88,000.0	63*	46	267	99
14	5,200.0	88,000.0			50*	148		
16	25,000.0	147,000.0			53*	162		
18	51,000.0	202,000.0	184,000.0	137,000.0	118	267	667	571
20	85,000.0				164			
22	111,000.0				316			
24	132,000.0	226,000.0	216,000.0	127,000.0	480	2250	157	−55

* Generation time during logarithmic growth period.

A CHEMICAL-BIOLOGICAL STUDY OF ESCHERICHIA COLI AND THREE OF ITS ROUGH VARIANTS[1]

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While working on the general subject of filtrability, certain variants of *Escherichia coli* were isolated which are referred to in this paper as *E. coli*-R-27, *E. coli*-R-32, and *E. coli*-R-G. The normal smooth parent strain is designated as *E. coli*-S. Each of the variant organisms formed a colony which was rough in appearance and grew in a characteristic manner when inoculated into broth. The letter "G" signifies only that the colony is small. The rate of multiplication of the parent and variant forms was determined to be the same.

The first section of the paper deals with the ability of these four forms to utilize the salts of organic acids as sources of energy. This is followed by a comparative study of the decomposition of glucose, and of the unique behavior of one of the variants in respect to this carbohydrate. The results of these experiments suggested the work which is expressed in the glucose utilization curves. The effect of the organism when injected into the animal body is also tested, and a sound explanation for the mechanism of action is given.

UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE PARENT CULTURE AND THE VARIANT FORMS

Using a synthetic medium, the ability of the four forms of the colon bacillus to utilize the carbon of certain organic acids as a

[1] A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

source of energy was studied comparatively. Terminal acidity was measured by the buffer system of Kolthoff (1925). Koser (1923) has conclusively proved that no growth will take place without a source of carbon such as an organic acid, glucose, or lactose. Pesch (1921) employed sodium tartrate and showed that five proven strains of *E. coli* grew well on it, but when sodium citrate was substituted, the same strains showed no growth even after 72 hours. Results are tabulated in table 1.

TABLE 1

Growth, gas production, and final pH of E. coli-S and variants cultured on a synthetic medium containing the sodium salts of organic acids

ACID	E. COLI-S		E. COLI-R-27		E. COLI-R-32		E. COLI-R-G	
	Growth	Final pH	Growth	Final pH	Growth	Final pH	Growth	Final pH
Formic	2+	8.0	2+	8.2	2+	8.2	2+	8.2
Acetic	4+	7.8	1+	6.6	2+	6.8	1+	6.7
Propionic	2+	7.6	1+	7.6	2+	7.6	1+	7.6
Butyric	1+	7.6	1+	7.6	1+	7.6	1+	7.6
Lactic	v. h.*	8.2	2+	8.0	3+	7.6	2+	7.6
Malic	3+	8.2	2+	8.2	1+	8.2	1+	8.2
Oxalic	1+	7.5	b. t.*	7.5	1+	7.6	1+	7.6
Valerianic	b. t.	7.6	1+	7.6	2+	7.6	1+	7.6
Tartaric	1+	7.5	2+	7.6	2+	7.5	2+	7.5
Citric	1+	7.5	1+	7.6	2+	7.6	2+	7.6
Benzoic	1+	7.5	1+	7.6	1+	7.5	1+	7.5
Salicylic	b. t.	7.4	b. t.	7.5	2+	7.5	1+	7.5

Initial pH was in each instance 7.6.
Proliferation was observed in Erlenmeyer flasks.
The Smith fermentation tube was used to estimate gas production.
No gas was formed.
* v. h. = very heavy; b. t. = barest trace.

Table 1 shows marked differences in ability to use the carbon of organic acids as a source of energy in the cases of acetic, lactic, malic, tartaric, citric and salicylic acids. With the first three acids the parent organism showed the greatest development, this being especially true of lactic acid. The last three were more readily utilized by one or more of the variants.

ACTION OF E. COLI-S AND THE THREE VARIANTS ON GLUCOSE AND LACTOSE

The production of acid and gas by the four forms was compared in broth, initial pH 7.6, containing 2 per cent of glucose; a parallel series contained the same amount of lactose. The volumes of gas formed are expressed in centimeters measured from the tops of the closed arms of fermentation tubes, uniform in size. The experiment was done in triplicate, readings being made at the end of 24, 48, and 72 hours. The final pH was 5.2 in all cases where growth occurred. The data are recorded in table 2.

TABLE 2

Gas production in sugar broths

(Heights in centimeters of gas columns in Smith tubes)

BROTH	TUBE	CULTURE											
		S			R-27			R-32			R-G		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Glucose	1	5.0	6.5	7.0	3.0	3.7	3.7	0	0	0	0.5	3.8	3.8
	2	5.5	6.0	6.3	—	—	—	0	0	0	2.0	4.6	4.8
	3	4.7	5.8	6.1	0.5	1.5	2.0	0	0	0	0.5	3.5	4.0
Lactose	1	1.6	2.5	3.0	*	0.3	1.1	*	*	1.2	0	0	0
	2	4.9	5.3	5.5	*	0.3	1.0	*	*	0.3	0	0	0
	3	4.0	4.5	4.8	*	0.5	1.7	*	0.3	2.0	0	0	0

— = no growth, due probably to faulty inoculation; 0 = heavy growth but no gas; * = first evidence of gas.

These results show that in broth containing glucose *E. coli*-R-32 formed no gas in contrast to the normal S form and the other variants. In broth containing lactose, *E. coli*-R-G formed no gas while the other two variants produced much less than the parent strain. The non-production of gas in glucose broth by *E. coli*-R-32 was studied further.

FURTHER STUDY OF THE ACTION OF E. COLI-R-32 ON GLUCOSE

Modification in the gas-producing power of *E. coli* through chemical means has been noted by Penfold (1911), Harden and

Penfold (1912), and Revis (1912). Through the use of sodium chloracetate, Grey (1913) produced a strain unable to form acetaldehyde from glucose. Lommel (1926) has recorded the loss of ability of this bacillus to attack lactose as the result of the action of malachite green. Pakes and Jollyman (1901) proved formic acid to be the immediate precursor of carbon dioxide, more than 80 of their strains being able to form carbon dioxide through the decomposition of this acid.

Inoculation of the four organisms was made into fermentation tubes containing (1) plain broth, (2) 2-per-cent glucose broth, (3) peptone water containing sodium formate and (4) plain broth

TABLE 3

Growth and gas production by E. coli-S and variants in glucose and sodium formate broths and peptone water

	CULTURE							
	S		R-27		R-32		R-G	
	Growth	Gas production	Growth	Gas production	Growth	Gas production	Growth	Gas production
Plain broth	+	0	+	0	+	0	+	0
Glucose broth	+	+	+	+	+	0	+	+
Na formate peptone water	+	+	+	sl.*	+	0	+	sl.
Na formate broth	+	+	+	+	+	0	+	sl.

* sl. = slight amount.

containing 1-per-cent Na formate. The following is the composition of the peptone water: (*a*) 2 per cent peptone, (*b*) 0.5 per cent sodium formate, (*c*) 1 per cent sodium formate. After sterilization and incubation for sterility, 8 tubes were inoculated in duplicate with each of the four strains. The readings were taken at the end of 72 hours, the results being recorded in table 3.

A careful analysis of the preceding data will show that those organisms which were able to form gas from glucose broth also formed it from the media containing sodium formate, although not necessarily to the same extent. The one strain which was thoroughly consistent in its action was *E. coli*-R-32.

With this information, the next step was to develop a technique for the analysis of formic acid, and the method of titration with $KMnO_4$ (Ost and Klein, 1908) was selected. Preliminary experiments showed that acetic acid caused no interference in the range studied.

Technique of the experiment. Six flasks were prepared in the following manner: 10 grams of Witte's peptone were boiled with tap water, and after cooling, 20 grams of glucose and 10 grams of pure calcium carbonate were added. The volume was then made up to 1 liter, after which the resulting mixture was sterilized at 110°C. for 30 minutes. This is essentially the medium used by Harden (1901) under somewhat similar conditions. After incubation for sterility, these flasks were inoculated in triplicate with *E. coli*-S and *E. coli*-R-32. At the end of 4 days' incubation at 38°C. the flasks were analyzed in the following manner:

The contents of a flask was first acidified with concentrated H_2SO_4, and then distilled to carry over volatile acids such as formic and acetic. The last portion was steam-distilled until tests showed that no further acids were coming over. The distillate was then made alkaline with sodium carbonate solution, evaporated to a convenient volume, and finally titrated hot with N/10 $KMnO_4$. The absence of higher acids such as butyric and propionic was indicated by qualitative tests. The titration data follow:

	Volume of N/10 $KMnO_4$ used cc.
1. *E. coli*-S	56.10
2. *E. coli*-S	57.55
3. *E. coli*-S	56.75
1. *E. coli*-R-32	114.35
2. *E. coli*-R-32	114.90
3. *E. coli*-R-32	113.85

This experiment shows that, even in the case of the S form of the organism, there is undecomposed formic acid when growth of the germs has ceased, but that in the case of the rough variant there is much more. It seems incontrovertible that in the case of *E. coli*-R-32 the reason for its failure to produce gas from glucose

FIG. 1. E. COLI-S

All photographs taken at magnification 10× from plain agar plates, 36 hours incubation.

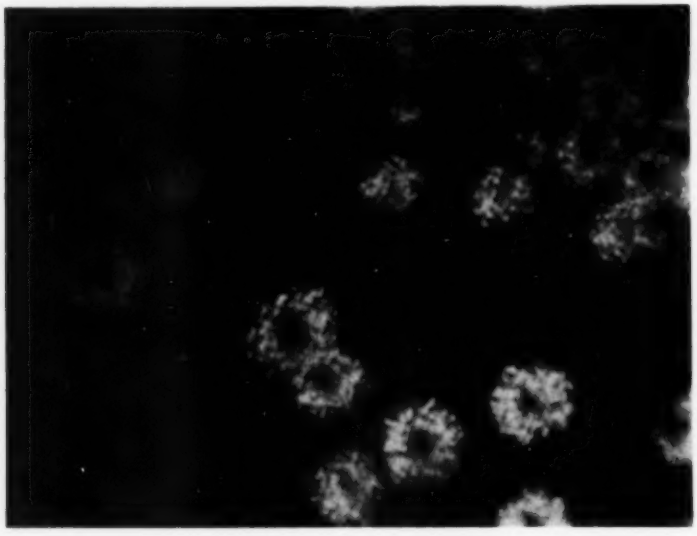

FIG. 2. E. COLI-R-27

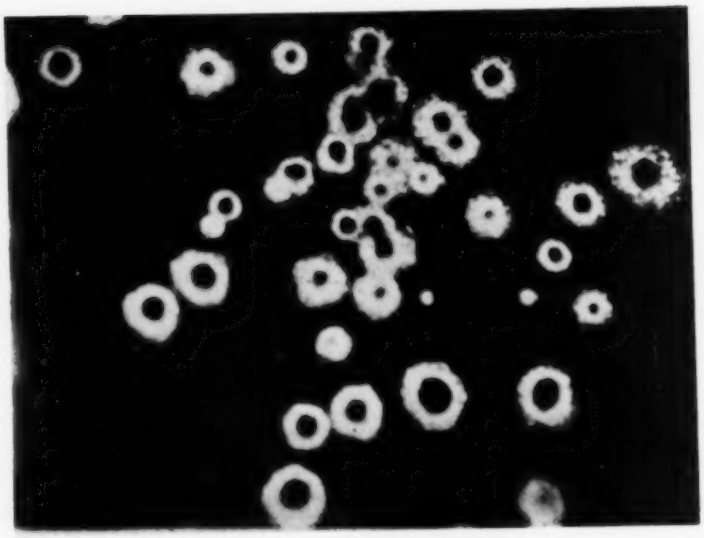

FIG. 3. E. COLI-R-32

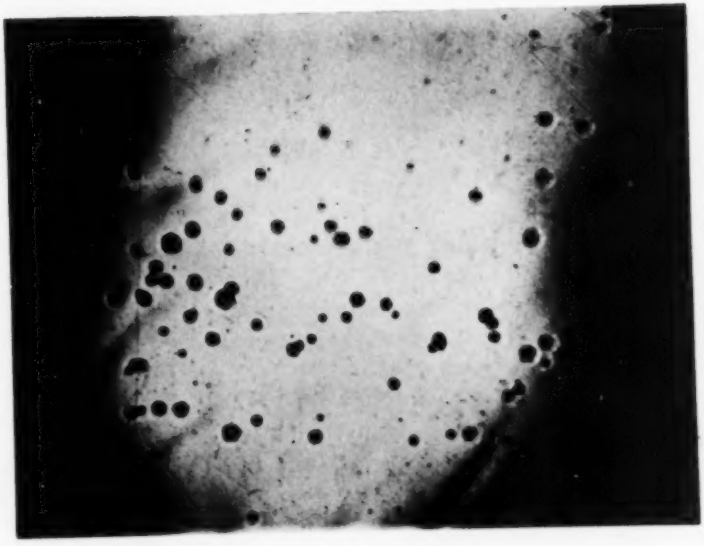

FIG. 4. E. COLI-R-G

is its inability to utilize formic acid. Penfold (1911) worked extensively on the general relation of the power of micro-organisms to form gases and their ability to decompose formates. He isolated a modified strain of *E. coli* by growth in the presence of sodium mono-chloracetate, and demonstrated that this variant formed acid but no gas from glucose, fructose, and lactose, although it still yielded gas from the alcohols used and from formates. Harden and Penfold (1912) later worked with this modified strain, proving that the main difference between the actions of the strains lay in the end products of fermentation,

TABLE 4

The utilization of glucose by E. coli-S when added to nutrient broth in various concentrations (average values)

NUMBER OF TESTS	VOLUME OF BENEDICT'S SOLUTION USED	ORIGINAL CONCENTRATION OF GLUCOSE	VOLUME OF FILTRATE USED FOR GLUCOSE DETERMINATION	FINAL CONCENTRATION OF GLUCOSE	GLUCOSE UTILIZED
	cc.	*mgm. per cc.*	*cc.*	*mgm. per cc.*	*per cent*
5	25.00	55.60	0.90	55.60	00.00
5	25.00	45.07	1.17	42.78	5.04
5	25.00	31.25	1.82	27.48	12.06
5	25.00	23.04	2.67	18.74	18.71
5	25.00	13.77	4.85	10.31	25.13
5	25.00	10.10	7.66	6.53	35.37
2	55.00*	6.50	2.00	3.23	50.35
2	55.00*	5.12	3.00	1.13	78.02

* Bang's solution used.

those from the modified strain containing less hydrogen and carbon dioxide and more formic acid than those from the normal strain. The atypical strain also made more lactic[2] acid than the normal strain and less alcohol and acetic acid.

GLUCOSE UTILIZATION BY E. COLI-S AND E. COLI-R-27

The utilization of glucose by *E. coli*-S and *E. coli*-R-27 was studied in a broth solution containing different amounts of the

[2] Neuberg and Gorr (1925) state that "lasst man das *Bacterium coli* auf Methylglyoxal einwirken, so Erfolgt, wie gesagt, die Umwandlung in Milchsäure quantitativ." This should be tested with respect to the variants of *E. coli*.

carbohydrates (tables 4 and 5). The concentrations of the solutions are expressed in milligrams of glucose per cubic centimeter, and these values are plotted (in fig. 5) against the per cent of glucose utilized by the organism. The concentrations of sugar ranged from those so low that fermentation was practically complete to values so high that no measurable amount was decomposed. Benedict's method of analysis was used when practicable, but in the extremely low dilutions this was replaced by the micro-method of Bang (1913). A uniform correction for evaporation, previously determined by analysis of a large number of samples, was applied.

TABLE 5

The utilization of glucose by E. coli-R-27 when added to nutrient broth in various concentrations (average values)

NUMBER OF TESTS	VOLUME OF BENEDICT'S SOLUTION USED	ORIGINAL CONCENTRATION OF GLUCOSE	VOLUME OF FILTRATE USED FOR GLUCOSE DETERMINATION	FINAL CONCENTRATION OF GLUCOSE	GLUCOSE UTILIZED
	cc.	*mgm. per cc.*	*cc.*	*mgm. per cc.*	*per cent*
5	25.00	62.5	0.85	58.8	3.8
5	25.00	41.66	1.26	39.56	4.27
3	25.00	27.03	2.02	24.87	8.00
3	25.00	17.86	3.36	14.88	16.66
3	25.00	12.50	5.48	9.12	27.00
2	55.00*	8.92	1.50	3.52	60.36
2	55.00*	5.83	2.00	0.86	85.35

* Bang's solution used.

BIOLOGY AND BIOLOGICAL APPLICATIONS

A. Effect on the blood sugar of the injection of E. coli-S and the three rough variants

In this experiment, the term blood sugar means reducing sugar or glucose. Zeckwer and Goodell (1925) injected killed cultures of *E. coli* suspended in physiological salt solutions into rabbits *via* the ear vein, and found that the content of blood sugar began to rise almost immediately, reaching a maximum in less than two hours and returning to normal in about four hours. Later (1925) the same authors proved that during bacterial

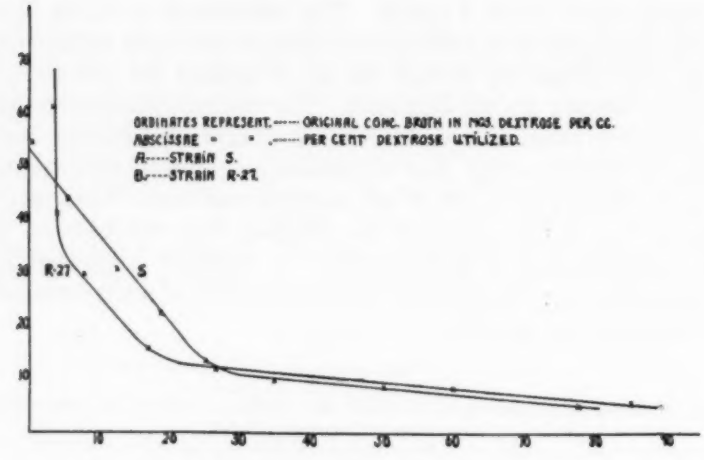

FIG. 5. GLUCOSE UTILIZATION CURVES

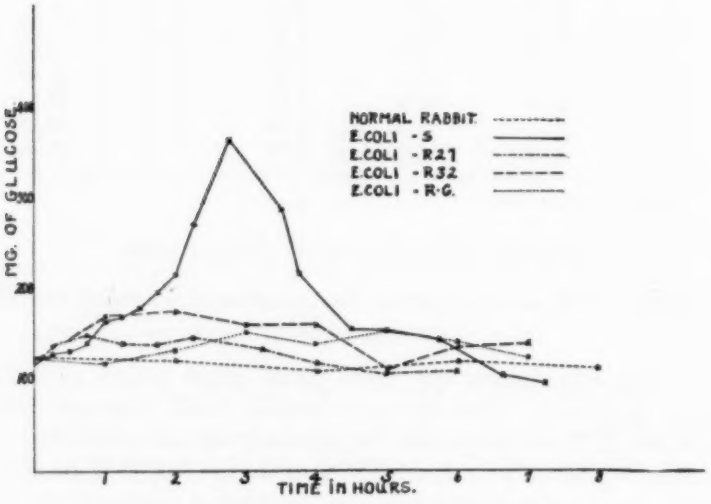

FIG. 6. MILLIGRAMS OF GLUCOSE PER 100 CC. BLOOD

anaphylaxis there was a gradual rise in the blood sugar which attained an extremely high level at the time of death. Delafield (1932) obtained practically identical results.

The procedure was to inoculate into the ear vein of a rabbit a certain amount of the young broth culture of the organism. The basis of the amount was enough of *E. coli*-S to cause the death of the animal in 24 hours. An equal amount of the other organ-

TABLE 6

Effect on the blood sugar of rabbits of the injection of broth cultures of E. coli-S and variants

INTERVAL AFTER INJECTION	NORMAL CONTROLS		COLI-S	R-27	R-32	R-G
	1*	2*				
minutes	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
00	138	117	120	117	127	127
15			124	134		
45			146	150		
60			170		177	122
75			172	141		
105			199	141		
120	148	101	221		180	138
135			276	150		
165			374			
180					166	157
240	127	101		122	166	143
270			159			
300			159	108	119	157
360	118	134		113	141	145
420					146	127
435			102			
480	101	134				

* Average is plotted as one curve.

isms was used, although it had been demonstrated many times that the variant forms are entirely harmless for rabbits. For the measurement of the blood sugar, the method of Hagedorn and Jensen (1922–23) was used. The data are presented in both tabular and graphic form.

These results show a marked difference in the reaction of the animal body to the S and R forms. Within three hours of the

injection of the S form, the blood sugar content had risen from 120 to 372 mgm. per 100 cc. of blood. Concurrently with this change, the rabbit became increasingly restless and died the same evening.

The reactions to the variants show considerable similarity and suggest a close relationship between the R forms. The slight increases in blood sugar are certainly due to the injection and not to normal variation, for a comparison with the two normal rabbits shows that this value is not changing greatly. None of the rabbits which received the injection of any of the variant forms showed any ill effects at any time.

It is therefore evident that the increase in the blood sugar must be due to a toxin formed by the normal S form and not at all, or at most in very small amounts, by the rough variants. Death of the animal in this, and more especially in other instances in which larger amounts of the broth culture have been used, has occurred too quickly to be ascribed to multiplication of the bacteria.

B. Pathogenicity for animals of E. coli-S and the three rough variants

The fact has been established that the parent organism when injected intravenously into rabbits produces death in a very short time, while similar injections of the other three forms leave the animals apparently unaffected. Through the injection of filtrates as well as of washed masses of cells which had been frozen and thawed repeatedly, it was conclusively demonstrated during this work that the lethal agent is an exo-toxin. In the dog, Denys and Sluyts (1894) proved the toxin of *E. coli* to have an intense action on the mucosa of the digestive tract. Roger (1894), using graphical methods, showed that the toxin of this organism made the heart less irritable. Vida (1905) believed that *E. coli* possessed only an endo-toxin while Vincent (1925) insisted that it possessed both an endo- and an exo-toxin. Steinberg and Ecker (1926) obtained from young cultures a toxic substance which proved to be the lethal factor when injected into rabbits. Smith (1927) stated that the offensive weapon of the organism appears to be a soluble, diffusible toxin.

In order to determine the manner in which the toxin of the normal S form caused death in rabbits, many of these animals were autopsied and the organs examined microscopically. The picture was invariably the same—extensive pigmentation in the liver but with the real significant pathology to be found in the lung. Here, the normal lung structure was almost obliterated, and the few alveolar spaces which remained were tense and distorted. These spaces were greatly dilated and engorged with red and white blood cells, giving the appearance of extensive hemorrhage. Many spaces had been obliterated by the presence of the blood cells in them as well as by pressure from the outside. Death was due to the fact that the respiratory membrane of the lung had been made suddenly permeable to the blood cells through the action of the bacterial toxin. This explains the death of the animal with symptoms of marked dyspnoea. This matter warrants much further investigation.

SUMMARY

Three rough variants of *Escherichia coli* have been developed which possess marked differences in comparison with each other and with the parent strain. As a source of energy, acetic, lactic and malic acids are more readily utilized by the parent cell, while tartaric, citric and salicylic acids are more readily utilized by one or more of the variants.

In broth containing two per cent glucose, one of the variants, *E. coli*-R-32, is unique in that there is an absence of gas formation with an end reaction which is acid. This is proved to be due to the inability of this cell to decompose formates.

With respect to the decomposition of glucose, it is demonstrated that *E. coli*-R-27, the most typical of the rough variants, has a less vigorous enzyme, or set of enzymes, than the parent cell.

The difference in the action of the normal strain and the variants on the animal body is marked. The injection of a lethal dose of *E. coli*-S into the ear vein of a rabbit causes a sudden rise in the blood sugar, and at the same time brings about the death of the animal in a few hours. The variants under identical conditions are almost without effect.

It is believed that the fatal agent in the action of the normal form is a soluble exo-toxin which produces death by making the walls of the lung capillaries suddenly permeable to the cellular and non-cellular elements of the blood.

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CONCERNING THE HABITAT OF STREPTOCOCCUS LACTIS

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The first streptococcus studied was *Streptococcus lactis*, which was discovered and isolated by Lister (1873, 1878) and named by him *Bacterium lactis*. Because of its technical importance this organism has been extensively investigated, but, notwithstanding the large body of scientific knowledge which has been accumulated concerning its biological properties and practical uses, the natural habitat of *Streptococcus lactis* has long been a moot question.

While utensils have been accepted as probably the most common immediate source of this organism in milk, such fortuitous objects on which growth may occur do not represent a true habitat in an ecological sense. In the older literature are to be found many claims of the occurrence of *Streptococcus lactis* in the udders, mouths and feces of cows, but such investigations were conducted before proper differential tests were at hand for the identification of *Streptococcus lactis* and its separation from other closely related types, especially *Streptococcus fecalis*.

It has long been amply established that *Streptococcus lactis* is not a normal inhabitant of the bovine udder. (Rogers and Dahlberg, 1914; Sherman and Hastings, 1915; Evans, 1916; Sherman and Albus, 1918; Orla-Jensen, 1919; Ayers and Mudge, 1922.) Although Esten (1909) reported the finding of *Streptococcus lactis* (*Bacterium lactis-acidi*) in the mouths and feces of cows, and McGuire (1915) claimed *Streptococcus lactis* to be a normal inhabitant of cow feces, Ayers and Mudge (1923), with more modern methods of study, did not find this organism among the characteristic streptococci of the bovine mouth,

throat or feces. While Esten used the characteristic reducing action in litmus milk as the criterion for *Streptococcus lactis*, his methods would not differentiate this organism from that group of fecal streptococci typified by *Streptococcus fecalis*. (Orla-Jensen, 1919; Sherman and Stark, 1931, 1934.)

METHODS

In the examination of such substances as ears of corn (in the husks), pods of peas, heads of cabbage and lettuce, etc. aseptic methods were employed to obtain samples from their interiors.

Samples of fresh corn, young corn silks, frozen fresh corn, corn meal, wheat flour middlings, dried navy beans, and young cabbage heads were placed in sterile distilled water and held at room temperature for twenty-four hours or longer. When a stained preparation made from such an infusion indicated that Gram-positive cocci in pairs or chains predominated, the sample was plated on milk agar. If Gram-positive cocci in pairs or chains failed to predominate but were observed in small numbers, the material was inoculated in serial dilutions into tubes of litmus milk. After incubation, milk agar plates were made from the highest dilution in litmus milk which showed reduction before curdling. From the milk agar plates pure culture isolations were made.

When examining materials such as lettuce, which contained small numbers of streptococci in the presence of large numbers of Gram-negative rods, it was found desirable to add the material to sterile water containing 10 per cent of sterile litmus milk. This medium was held until sufficient acid was produced to retard the growth of Gram-negative rods and accelerate overgrowth by the streptococci. When this condition prevailed serial dilutions were made into litmus milk tubes, isolations being made by plating after the streptococci gained the ascendency in the milk cultures.

Samples from the mouths and throats of cows, taken by means of sterile swabs, were placed in sterile water containing litmus milk and held at 10°C. for a few days to prevent the growth of *Streptococcus bovis* (and its relatives), normally present in large

numbers in the cow's mouth, and to permit its overgrowth by other streptococci present in smaller numbers. Later, these samples were incubated at 30°C. until enough acid was produced to inhibit or partially destroy the Gram-negative rods present. From milk agar plates made from this material, streptococci (but not *Streptococcus lactis*) were readily isolated. Additional isolations were made after further culturing in litmus milk.

The samples of feces, soil, and other non-vegetable materials were placed in sterile water containing litmus milk, and after incubation at room temperature, transfers were successively made through litmus milk cultures as outlined above, in attempts to isolate *Streptococcus lactis*.

The streptococci isolated were tested for their ability to grow at 10°C. and 45°C., action in litmus milk, action on blood agar, liquefaction of gelatin, production of ammonia from peptone, production of carbon dioxide from glucose, limiting pH in glucose broth, and action on eleven carbohydrates and related test substances. As these methods are all now more or less standard procedures in the study of streptococci, no special description of the techniques is called for. Because of its misapplication by a few workers, however, it is desirable to say a word about the testing of bacteria for their temperature limits of growth: not only is it necessary that the incubator temperature be accurate, but the test cultures should be adjusted immediately after inoculation to the incubation temperature in a water bath, and incubation should be carried out in sealed tubes or otherwise so as to preclude a cool layer at the surface of the culture due to excessive evaporation.

EXPERIMENTAL FINDINGS

In all, 200 cultures which were identified as *Streptococcus lactis* have been isolated from various plant sources. Both the grains and silks from fresh corn at the proper stage for human consumption were found in every instance tested to contain *Streptococcus lactis*. Only one sample each of frozen corn and corn meal was tested, but *Streptococcus lactis* was isolated from each of these. Several samples of dried navy beans were tested and found in each

case to contain *Streptococcus lactis*, which could be isolated with ease. It was also obtained from cabbage, head lettuce, garden peas, and wheat middlings, but because of the presence of larger numbers of other types of bacteria the isolation of *Streptococcus lactis* from these sources was more difficult.

The characteristics of the 200 cultures isolated from plants, together with their sources, are given in table 1. Included in the table for comparative purposes are the characteristics of 35 cultures of *Streptococcus lactis* which were recently isolated from milk and milk products. As may be noted from the table, the characteristics of the organisms from plants agree with those

TABLE 1

Characteristics of cultures of Streptococcus lactis from plants and from milk

SOURCE	NUMBER OF CULTURES	GROWTH AT 10°C.	GROWTH AT 45°C.	LITMUS MILK REDUCED BEFORE CURDLING	BETA TYPE OF HEMOLYSIS ON BLOOD AGAR	FINAL pH OF 4.2 TO 4.0 IN GLUCOSE BROTH	AMMONIA PRODUCED FROM PEPTONE	CARBON DIOXIDE PRODUCED FROM GLUCOSE	LIQUEFACTION OF GELATIN	ARABINOSE	XYLOSE	GLUCOSE	LACTOSE	MALTOSE	SUCROSE	RAFFINOSE	INULIN	GLYCEROL	MANNITOL	SALICIN
Corn	82	+	−	+	−	+	+	−	−	+	+	+	+	+	±	−	−	−	+	+
Navy beans	48	+	−	+	−	+	+	−	−	±	±	+	+	+	±	−	−	−	±	+
Cabbage	20	+	−	+	−	+	+	−	−	−	+	+	+	+	−	−	−	−	+	+
Wheat	19	+	−	+	−	+	+	−	−	−	+	+	+	+	±	−	−	−	±	+
Garden peas	18	+	−	+	−	+	+	−	−	−	+	+	+	+	−	−	−	−	−	+
Head lettuce	13	+	−	+	−	+	+	−	−	−	+	+	+	+	+	−	−	−	+	+
Milk	35	+	−	+	−	+	+	−	−	−	±	+	+	+	±	−	−	−	±	+

obtained from milk and with the properties of *Streptococcus lactis*, as this species or group has been described and limited by recent authorities.

While negative findings in a study of this type should be accepted with some reserve, it is interesting to note the sources from which we failed to isolate the organism. *Streptococcus lactis* was not obtained from: mature corn, 12 samples; aged heads of cabbage, 2 samples; lima beans, 2 samples; alfalfa, 2 samples; soil, 12 samples; intestines of earth worms, 6 samples; mouths and throats of cows, 4 samples; bovine feces, 12 samples; human feces, 7 samples.

It should be noted that *Streptococcus fecalis*, which has been very generally confused with *Streptococcus lactis*, was obtained from the mouths and throats of cows, bovine feces and human feces.

From the data given in table 1 it is seen that all of the 235 cultures from plants and milk agree perfectly with the standard descriptions of *Streptococcus lactis*. Without exception, they were found to have the following characteristics: Growth took place at 10°C.; there was no growth at 45°C.; litmus in milk cultures was reduced before curdling took place; true hemolysis of the beta type was not produced in blood; a low final pH was produced in glucose broth; ammonia was produced from peptone; carbon dioxide was not produced from glucose; gelatin was not liquefied; glucose, maltose, lactose and salicin were fermented; but raffinose, inulin and glycerol were not fermented.

Fermenting and non-fermenting strains were found with arabinose, xylose, sucrose and mannitol, which finding is in keeping with the results of all workers who have used these test substances with the *Streptococus lactis* group. It should be noted also that strains which do not ferment maltose or salicin are well known, but were not encountered in the present study. Likewise, Ayers, Johnson and Mudge (1924) have reported strains of *Streptococcus lactis* which do not produce ammonia from peptone, while Hammer and Baker (1926) and others have recognized varieties based on characters not used in this study. In connection with the variable characters found in the group, it should be mentioned that while *Streptococcus lactis* has never been found to produce true hemolysis of the beta type on blood agar, strains which give either the alpha (usually weak) or gamma reaction are common, and both types were represented in the collection used in this study. In addition, some strains of *Streptococcus lactis* cause a slight hydrolysis of starch.

While all agree that individual strains of the *Streptococcus lactis* group vary in their respective actions on arabinose, xylose, sucrose and mannitol, for our present purpose it is desirable to show whether or not the cultures obtained from plants correspond with those from milk and milk products with respect to these characters. These data are assembled in table 2.

It is seen that the cultures fall into nine groups based upon their actions on these four test substances. The plant strains are found in eight groups, while the small collection of only 35 cultures from milk fall in six groups, five of which coincide with groups from plants. Three groups (1, 3 and 4) are represented by plant strains but not by any of the milk cultures included in this collection. However, reference to the published data of other workers reveals cultures of *Streptococcus lactis* from milk or milk products which fit into these groups: Group 1, Anna Orla-Jensen and Hansen (1932); group 3, S. Orla-Jensen (1919); and group 3, S. Orla-Jensen (1919), and Anna Orla-Jensen and Hansen (1932).

TABLE 2

Cultures of Streptococcus lactis from plants and milk grouped according to their abilities to ferment arabinose, xylose, sucrose and mannitol

GROUP	ISOLATED FROM	ARABINOSE	XYLOSE	SUCROSE	MANNITOL
1	Corn, navy beans, milk*	+	+	+	+
2	Lettuce, wheat, milk	−	+	+	+
3	Corn, milk*	+	+	−	+
4	Cabbage, wheat, milk*	−	+	−	+
5	Milk	−	−	+	+
6	Wheat, milk	−	+	+	−
7	Navy beans, milk	−	−	−	+
8	Garden peas, wheat, milk	−	+	−	−
9	Navy beans, milk	−	−	−	−

* Milk cultures in groups 1, 3 and 4 obtained from data of other workers.

Although somewhat aside from the present problem, it is interesting to note that of the sixteen possible groupings with the use of the above four test substances, nine groups are represented in the collection of 235 cultures used in this study. As a matter of fact we have been able to find representatives of the remaining seven groups in the data of other workers. In other words, those who are inclined to divide the *Streptococcus lactis* group into additional species on the basis of slight variations would obtain sixteen "species" by the use of these four test substances. If the other known variable characters were included, the resulting number of possible "species" would become absurd.

DISCUSSION

The data presented in this paper are sufficient evidence, we believe, that *Streptococcus lactis* occurs on plants and that, therefore, this source is the most probable one from which it finds its way into milk. This finding is not surprising, as doubtless many others have also suspected that this organism is associated with plants, rather than animals, in nature. There have been many reports of the occurrence of *Streptococcus lactis* among the lactic acid bacteria present in fermenting vegetable materials, but such a finding is no more remarkable, nor ecologically significant, than its occurrence in sour milk.

Hüttig (1931) claimed the conversion of *Streptococcus lactis* into *Bacterium herbicola*, and *vice versa*, by heating the cultures at 58°C. for twenty minutes, combined with aging and other manipulations. He considered *Streptococcus lactis* to be a dissociative form of *Bacterium herbicola*. While not to be interpreted as a reflection upon the interesting results of Hüttig, our observations while isolating *Streptococcus lactis* from plant materials may have some pertinence in this connection. These two organisms were frequently observed growing together in mixed cultures on agar plates made from plant infusions, and not infrequently in the same well-isolated colony. Sometimes, discrete colonies of *Streptococcus lactis* were observed growing within and up through the colonies of *Bacterium herbicola*. However, we experienced no difficulty in separating the two organisms by reisolation, and it should be emphasized that we observed no such radical dissociation among the pure cultures of *Streptococcus lactis* which were used in the present investigations.

Although it appears to be established that *Streptococcus lactis* occurs on plants, conclusions concerning the natural habitat of an organism should not be lightly drawn. *A priori*, one might expect upon the discovery of a true habitat of a bacterium to find there the organism in large or fairly abundant numbers. We have no evidence that this is true in the case of *Streptococcus lactis* on plants. On the other hand, many bacteria are found in only small numbers in what we believe to be their natural habitats, as, for examples, certain soil and water forms and those

which occur on the skin, within the udder, etc. In the course of evolution, many organisms have found habitats under environmental conditions which serve admirably for the preservation of the species but place rigid restrictions upon reproduction. In the present case, we feel that the data presented are suggestive but do not warrant dogmatic conclusions concerning the true habitat of *Streptococcus lactis*.

SUMMARY

Streptococcus lactis has been repeatedly isolated from certain plants but not from all plants examined.

It was not found in the mouths nor throats of cows, bovine feces, human feces, nor in soil.

It is suggested that plants may represent the natural habitat of *Streptococcus lactis*.

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THE RATE OF GROWTH OF RHIZOBIA

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In connection with another problem, we have had occasion to make some growth rate studies of a number of strains of the nodule bacteria of the leguminosae; and, since the literature of the rhizobia appears to be peculiarly deficient in such data, it is perhaps worthwhile to record briefly the results. Since our particular interest was in the relative rates of growth of the various strains of a species, rather than their exact maxima, no claims are made for the accuracy of these data as representing maximum possible rates of growth of the rhizobia. The temperature used in these experiments (25°C.) may not be the optimum; and doubtless special media could be devised which would promote faster growth.

The cultures were grown in a liquid medium (pH 6.8) containing, per liter of distilled water:

	grams
Mannitol	10
Yeast extract (Bacto)	1
Ammonium nitrate	0.5
Potassium nitrate	0.5
Dibasic potassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.2

This medium, after sterilization, was added in 100 cc. portions, under sterile conditions, to 8-ounce bottles containing 25 cc. of 3 per cent washed agar and 5 per cent of calcium carbonate. The bottles containing the calcium carbonate suspended in agar had been previously sterilized and cooled so that the liquid medium remained above the agar layer. In our hands, this contact with the calcium carbonate-agar layer gave much better

growth in the liquid medium than was obtained in the same medium without this contact. The use of an excess of calcium carbonate directly in liquid media was discarded for this work because of the tendency for many of the cultures to produce a heavy, slimy growth so entangled with the calcium carbonate as to render accurate plate culture counts difficult to obtain. With the technique used, even, well-dispersed growths were obtained throughout the medium. With the exception of the added nitrates and a weaker yeast supplement, this culture solution is similar to the one especially recommended by Fred, Baldwin and McCoy (1932).

Tests were made with 17 cultures representing 3 species: *Rhizobium leguminosarum*, *R. meliloti*, and *R. trifolii*. We are indebted to Professors E. B. Fred, I. L. Baldwin and their associates of the University of Wisconsin for the cultures used. As indicated above, the organisms were grown at 25°C. in the liquid medium. Plate culture counts were made at hourly intervals over a sufficient time to cover the period of most rapid growth.

Since the generation times of the rhizobia are usually greater than one hour, the calculations of growth rates were made on the basis of a two-hour period or more, instead of for one hour as is the usual custom. In terms of generations, the procedure followed is approximately equivalent to the use of a one-hour period when the most rapidly growing species of bacteria are studied. Somewhat shorter generation times would have been obtained had the calculations been based on the one-hour maximum rate. Our other reasons for departing from the usual practice may not be sound, but as we do not wish to challenge, at the present time at least, the classical tenet that the bacterial growth curve is a smooth one during the period of most rapid reproduction, this point will not be discussed further. In the experiments with *Rhizobium leguminosarum* and *R. trifolii* the test cultures were inoculated from old cultures and hence went through the usual lag period, the generation times being calculated from periods of two or more hours between the tenth and eighteenth hours. As the *R. meliloti* test cultures were inoculated from young cultures, the lag period was substantially eliminated and the calculations

were made from counts obtained between the third and seventh hours. The results obtained are given in table 1.

From the data given in table 1, it is seen that generation times ranging from 1.25 to 3.64 hours were obtained. While in these tests average generation times of 2.1, 2.5 and 2.9 hours were obtained for *Rhizobium leguminosarum*, *R. meliloti* and *R. trifolii* respectively, the wide spread in the growth rates of the various strains of each species makes it doubtful if, in general, there is

TABLE 1

Maximum growth rates of 17 strains, representing 3 species, of Rhizobium at 25°C.

SPECIES	CULTURE	PERIOD*	GENERATION TIME
		hours	hours
Rhizobium leguminosarum	A-302	15–18	1.31
	F-325	12–18	1.69
	C-310	15–18	2.22
	E-312	15–18	2.44
	B-305	12–18	2.47
	D-311	12–18	2.51
Rhizobium meliloti	F-102	5–7	1.25
	A-131	5–7	2.07
	G-105	5–7	2.76
	E-103	5–7	2.79
	C-109	3–7	2.85
	B-101	5–7	3.23
Rhizobium trifolii	E-210	13–16	2.34
	B-201	10–16	2.64
	D-209	10–16	2.96
	A-202	10–16	3.14
	C-205	10–16	3.64

* See text for explanation.

any significant species difference in growth rate among these three groups. Even though exact generation time data are not available, it is well known that certain species, such as *Rhizobium Japonicum*, grow much more slowly in ordinary laboratory media than do those used in this study.

Again it should be noted that faster growth rates could probably be obtained, either with more favorable media or in actively aerated cultures.

SUMMARY

Growth rates of seventeen cultures of *Rhizobium leguminosarum* *R. meliloti* and *R. trifolii* were determined at 25°C. in a mannitol yeast-extract mineral salts broth. Under the conditions of these experiments, generation times of from 1.25 to 3.64 hours were obtained during the periods of most rapid growth.

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PROCEEDINGS OF THE LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH

UNIVERSITY OF WISCONSIN, OCTOBER 11, 1935

GROWTH STIMULANTS FOR CERTAIN RHIZOBIA. *W. B. Sarles and J. J. Reid*, Department of Agricultural Bacteriology, University of Wisconsin.

The rate of growth and total number of cells produced by *Rhizobium Japonicum*, *Rhizobium lupini* and the cowpea Rhizobium were found to be increased by adding potato extract to the usual yeast-water mannitol mineral salts agar. When 2 grams per liter of asparagine were added to this medium in place of the potato extract, some stimulation occurred, but the results were not as marked as those secured with potato extract. It is suggested that potato extract be added to media used to cultivate the slow-growing rhizobia.

GROWTH AND LONGEVITY OF RHIZOBIA ON AGAR CONTAINING VARIOUS ENERGY SOURCES. *J. J. Reid and W. B. Sarles*, Department of Agricultural Bacteriology, University of Wisconsin.

Mannitol and sucrose and a mixture of the two in a yeast-extract mineral salts agar medium have been compared as sources of energy for *Rhizobium meliloti* and *Rh. trifolii*. It was found that both species reached a maximum count in a shorter period of time on the sucrose medium, though no significant difference was found in total number of cells after an incubation period of 14 days. Counts made after storage of one year showed approximately the same number of living cells on each substrate. Use of sucrose with mannitol gave poorer results in every instance.

Infectivity was not influenced by substitution of sucrose for mannitol in the medium.

CORRELATION OF PATHOGENICITY AND VISCOSITY IN CULTURES OF PHYTOMONAS TUMEFACIENS. *T. O. Berge, A. J. Riker and I. L. Baldwin*, University of Wisconsin.

Differences have been detected in the viscosity of single-cell sister cultures of *Phytomonas tumefaciens* (Smith and Town) Bergey *et al.*, one of which was pathogenic on tomato at 28°C. and below but not at 30°C. and above, and the other of which was nonpathogenic on this host. A lower dyne value has been found with the duNöuy tensiometer for giant colonies of the pathogenic strain than for the non-pathogenic culture when grown on various agar media at 26°C. The viscosity of both cultures was considerably higher after several days at 32° than at 26°C.

Non-pathogenic cultures have been found to be more viscous after incubation for a number of days in certain liquid media than pathogenic cultures grown under the same conditions.

Since these differences in viscosity doubtless are associated with the character of the bacterial gums produced by

the cultures, it appears that these gums may be important in the pathogenicity of the organism.

DISSIMILATION OF GLYCEROL BY INTERMEDIATE BACTERIA OF THE COLON-AEROGENES GROUP. *Howard Reynolds and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames.

Braak (1928) and Werkman and Gillen (1932) reported on the dissimilation of glycerol by colon-aerogenes intermediates and found cultures (citrate-positive, V-P negative, M. R. positive) which fermented glycerol, anaerobically, in a medium of inorganic salts as a source of nitrogen-produced trimethylene glycol. The intermediary dissimilation of glycerol with *Citrobacter Freundii* has been studied by use of fixing reagents. Bisulphite causes the accumulation of pyruvic acid and acetaldehyde which have been identified as intermediary compounds. Under the same conditions or with peptone typical *Escherichia coli* and cultures of *Aerobacter* gave no detectable quantities of aldehyde or pyruvic acid. *C. Freundii* with dimethyl-dihydroresorcinol formed a deposit of an apparently pure crystalline compound which has been tentatively identified as acrolein.

Results with the fixation of pyruvic acid suggested the possibility of differentiating these colon-aerogenes intermediates. Colon-aerogenes cultures were grown in glycerol bisulphite broth and periodically tested for the formation of pyruvic acid. Results after forty-eight hours (37°C.): Fifteen cultures *Escherichia coli*—all negative; 57 intermediate cultures—44 positive; 20 *Aerobacter* cultures—17 negative.

THE ISOLATION AND POSSIBLE INTERMEDIARY RÔLE OF FORMALDEHYDE IN THE PROPIONIC ACID FERMENTATION. *H. G. Wood and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames.

Formaldehyde has been isolated from fermentations of glucose with certain cultures of *Propionibacterium* in the presence of dimethyl-dihydroresorcinol as a fixative. The formaldehyde dimedon compound has been identified by its melting point and the mixed melting point with authentic formaldimethone, also by preparation of its anhydride and determination of the melting points. The detection of formaldehyde suggests its function as an intermediary compound in the glucose dissimilation. Wood and Werkman recently found that the propionic acid bacteria utilize CO_2 during the dissimilation, of glycerol. In glucose fermentation, a similar utilization of CO_2 perhaps occurs with formaldehyde as an intermediary. Certain glucose fermentations have been found to yield large quantities of succinic acid with correspondingly small quantities of CO_2. Virtanen's proposal that the succinic acid is formed by a 4- and 2-carbon cleavage is adaptable to these results since succinic acid is produced independently of CO_2. It is significant that cultures yielding this type of dissimilation produce formaldehyde. It is suggested that the succinic acid is formed by a condensation of acetic acid and that the CO_2 yield is decreased by its utilization.

ACTIVATION OF THE LOWER FATTY ACIDS BY PROPIONIC ACID BACTERIA. *R. W. Stone, H. G. Wood and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames, Iowa.

Further redox potential studies with the propionic acid bacteria have been carried out on the lower fatty acids. Redox tubes equipped with a sidearm, platinum and agar electrodes, a gas inlet tube, and a gas outlet were employed; these were placed in a constant

temperature water bath at 37°C. *Propionibacterium arabinosum, P. pentosaceum,* and *P. Shermanii* were used.

Fatty acid dehydrogenases of *Propionibacterium* are not markedly affected by repeated washing of the cells, but are inhibited by aging of the cultures or washed suspensions. Formic acid showed some donation of hydrogen to methylene blue, but in a few cases also appeared to possess ability to act as a hydrogen acceptor. The activation of acetic acid to various acceptors takes place at pH 5.0 to 7.0. With nitrates activation is much stronger at acid pH values than at 7. Propionic acid readily donates hydrogen to available acceptors particularly at pH 6.0 to 7.0. This acid may go to lactic acid in fermentation under certain conditions. Butyric and valeric acids are not activated and may possess an inhibitory effect on dehydrogenase activity of propionibacterium.

OXIDATION-REDUCTION CHARACTERISTICS AND YEAST GROWTH INITIATION IN A MEDIUM CONTAINING SMALL AMOUNTS OF WORT. *D. R. Colingsworth and J. J. Reid,* Department of Agricultural Bacteriology, University of Wisconsin.

If grain wort is added to a synthetic medium to give a 1:100 wort dilution, growth initiation of *Saccharomyces cerevisiae* is obtained with the relatively small inoculum of 1 cell per cubic centimeter; a wort dilution of 1:1000 permits the growth initiation of yeast if the seeding is 1,000 cells per cubic centimeter; untreated medium requires an inoculum of 10,000 cells per cubic centimeter to insure growth initiation.

Addition of 0.02 per cent thioglycollic acid and 0.1 per cent sodium thiosulfate to the medium with 1:1000 wort dilution gave growth initiation with lower initial seedings of yeast than either medium containing only wort or reducing agent.

Potentiometric measurements of sterile medium with 1:100 wort dilution revealed that at the end of twenty days the potential was 50 mv. more positive than the potential of untreated, sterile medium. The potential of inoculated medium containing 1:100 wort was 200 mv. more positive after the twenty day period than inoculated wort-free medium.

CENTRAL PENNSYLVANIA BRANCH

STATE COLLEGE, PENNSYLVANIA, OCTOBER 19, 1935

A STUDY IN THE DIFFERENTIATION OF ESCHERICHIA COLI AND AEROBACTER AEROGENES BY THE BACTERIOSTATIC ACTION OF ORGANIC DYES. *Vincent A. Halbert,* Bucknell University, Lewisburg, Pennsylvania.

The purpose of this study was to differentiate between *Escherichia coli* and *Aerobacter aerogenes* for diagnostic purposes on the basis of the bacteriostatic action of various concentrations of organic dyes.

The base media used for this study consisted of standard extract agar with a pH value of 7.8. The dyes used, all of which were water soluble, were obtained from one source to provide standard quality of preparation.

The procedure consisted of pouring one half of the plate with plain extract agar and the other half with the dye infused agar. Streak inoculations of *Escherichia coli* and *Aerobacter aerogenes* were then made on the same plate, providing a comparison of the growth of the two organisms. The dye concentrations used were: 1:500, 1:1000, 1:3333, 1:5000, and 1:10,000. All plates were read after twenty-four hours incubation.

Dahlia B, Janus green B., Martius Yellow, Bensyl violet, and Aldehyde green exhibited differential inhibitory qualities between *Escherichia coli* and *Aerobacter aerogenes* as characterized by the rapid and luxuriant growth of the former and complete inhibition of *Aerobacter aerogenes*.

A Comparison of Ten Presumptive Test Media Used in the Detection of the Escherichia-Aerobacter Group in Milk. *Michael A. Farrell*, The Pennsylvania State College, State College, Pennsylvania.

In an investigation of 10 of the better known presumptive test media proposed for use in water and milk analysis, 66 samples of milk were planted into these media, using 3 dilutions of milk and 5 tubes of each dilution, followed by "Standard Methods" verification procedures, to determine which medium was the most sensitive in the detection of the *Escherichia-Aerobacter* group of bacteria in milk.

The results of this study showed brilliant-green lactose bile, fuchsin lactose broth and methylene-blue bromcresol-purple broth media superior to the others in that they detected colon group members in 53 per cent of the total number of samples examined. The remaining 7 media-standard lactose broth, buffered lactose broth, crystal-violet lactose broth, formate ricinoleate broth, gentian-violet lactose broth, MacConkey's peptone lactose broth and trypaflavine broth-were 20 per cent less efficient in the detection of this group than the above three.

Postmortem Bacteriology. *Henry F. Hunt*, The Geisinger Memorial Hospital, Danville, Pennsylvania.

The value of most postmortem examinations is enhanced tremendously if a careful bacteriologic study is made on the fluids and organs of the body; in many instances a sound conclusion as to the cause of the patient's illness and death can only be reached by such bacteriological research.

This paper is based on a study of 567 postmortem examinations. The percentage of positive postmortem blood cultures obtained was thirty per cent and in all but one case in which cultures of blood were positive, definite foci of infection were demonstrable.

From the uniformity of results, it is believed that postmortem bacteriology is reliable and if carried out carefully and as a routine, often leads to the solution of the exact cause of death.

Chromogens and History. *Roger D. Reid*, The Pennsylvania State College, State College, Pennsylvania.

A brief review of the effect of *Serratia marscesens* on history and the relation of that chromogen to the so-called "blood miracles."

The Biology of a New Cyclostage (the Critical R) of the Tubercle Bacillus. *Ralph R. Mellon*, Western Pennsylvania Hospital, Pittsburg, Pa.

This acid-fast organism has been observed to dissociate from both the normal virulent S form, and from the wholly saprophytic nonacid-fast variants of the tubercle bacillus. It has biological characteristics which distinguish it clearly from the so-called R forms of Kuhn, Petroff, and others. They appear as intermediates between this luxuriantly growing Critical R strain and the normal S form. The critical R is decidedly more saprophytic in its general cultural characters than the intermediate R-s; in addition, it produces a rather specific tuberculin. That is to say, this tuberculin reacts

positively in animals sensitized with the killed critical R-form; but the tuberculin from the virulent S-form does not react appreciably in these animals.

The fact that the critical R form has been dissociated from the nonacid-fast strains conventionally found in cases of human sarcoid of the skin, suggests that the curious epithelioid, *but non-caseating*, lesions of this tuberculous anomaly owe their origin to such strains. Reactions of the strain in animals tends to confirm this point of view, as far as the experiments have proceeded. The production of non-caseating and pyogenic lesions raises the question of the possible rôle of such strains in the normally induced immunity to tuberculosis.

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